

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
REQUEST FOR FILING APPLICATION UNDER RULE 1.53(b)**

Pursuant to 37 CFR 1.53(b), please file a ☒ continuation/☐ divisional
of the pending prior PATENT APPLICATION of:

Inventor: WINTER et al.

Serial No. 08/470,031

Filed: June 6, 1995

For: SINGLE DOMAIN LIGANDS, RECEPTORS COMPRISING SAID LIGANDS, METHODS FOR
THEIR PRODUCTION AND USE OF SAID LIGANDS AND RECEPTORS

Assistant Commissioner for Patents

Washington, DC 20231

Sir:

Atty Dkt.: 620-122

C# M#

Date: November 28, 2000

Group: 1636

Examiner: J. Ketter



This request for filing under Rule 53(b) is made by the following named inventor(s) (using the above-identified title):
Inventor(s): WINTER et al.

- ☒ Attached is a copy of a declaration from a prior application (37 C.F.R. § 1.63(d)).
☒ Priority is hereby claimed under 35 USC 119 based on the following foreign applications, the entire content of which is hereby incorporated by reference in this application:

Application Number		Country		Day/Month/Year/Filed	
1) 8826444.5	6) 8913900.0	1) UK	6) UK	1) 11/11/1988	6) 16/06/1989
2) 8906034.7	7) 8918543.3	2) UK	7) UK	2) 16/03/1989	7) 15/08/1989
3) 8909217.5	8) PCT/GB89/01344	3) UK	8) WO	3) 22/04/1989	8) 13/11/1989
4) 8911047.2		4) UK		4) 15/05/1989	
5) 8912652.8		5) UK		5) 02/06/1989	

- ☐ certified copy(ies) of foreign application(s) attached or
☐ already filed on _____ in prior appln. no. _____ filed _____
☒ already filed in PCT/GB89/01344 file d November 13, 1989

☒ The prior application is assigned to Medical Research Council.

☒ Power of Attorney has been granted to Thomas E. Byrne et al, Reg. No. 32,205 of Nixon & Vanderhye P.C., 1100 N. Glebe Rd., 8th Floor, Arlington, VA 22201.

☒ Address all future communications to: Nixon & Vanderhye P.C., 1100 N. Glebe Rd., 8th Floor, Arlington, VA 22201.

☒ Please amend the specification by inserting before the first line --This is a continuation of application Serial No. 08/470,031, filed 6/6/95, now pending, which is a divisional of Serial No. 08/332,046, filed 11/1/94; which is a continuation of Serial No. 07/796,805, filed 11/25/91, which is a divisional of Serial No. 07/580,374, filed 9/11/90, abandoned the entire content of which is hereby incorporated by reference in this application.--

☒ Petition filed in prior application to extend its life to insure copendency.

☒ The Examiner's attention is directed to the prior art cited in the parent application by applicant and/or Examiner for the reasons stated therein and return of an initialed copy of the attached PTO-1449 Form listing same, pursuant to MPEP §609, are requested.

☒ Please enter the attached and/or below preliminary amendment **prior** to calculation of filing fee:

PRELIMINARY AMENDMENT

- ☒ The entire disclosure of the prior application above-referenced is considered as being part of the disclosure of this new application and is hereby incorporated by reference therein.

FILING FEE IS BASED ON CLAIMS AS FILED LESS ANY HERewith CANCELED

Basic Filing Fee				\$	710.00
Total effective claims	32	- 20 (at least 20) =	12	x \$	216.00
Independent claims	3	- 3 (at least 3) =	0	x \$	0.00
If any proper multiple dependent claims now added for first time, add \$270.00 (ignore improper)				\$	0.00
SUBTOTAL					\$ 926.00
SECOND SUBTOTAL					\$ 926.00
TOTAL FEE ENCLOSED					\$ 926.00

Any future submission requiring an extension of time is hereby stated to include a petition for such time extension. The Commissioner is hereby authorized to charge any deficiency in the fee(s) filed, or asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our **Account No. 14-1140**. A duplicate copy of this sheet is attached.

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NIXON & VANDERHYE P.C.

By Atty: B. J. Sadoff, Reg. No. 36,663

Signature: _____

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

WINTER et al.

Atty. Ref.: 620-122

Continuation of Serial No. 08/470,031

Group: 1636

Filed: Herewith

Examiner: Ketter

For: SINGLE DOMAIN LIGANDS, RECEPTORS COMPRISING SAID LIGANDS,
METHODS FOR THEIR PRODUCTION AND USE OF SAID LIGANDS AND
RECEPTORS

* * * * *

November 28, 2000

Assistant Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Preliminarily amend the above-identified application as follows.

IN THE CLAIMS:

Amend the claims as follows.

Claim 8, line 1, delete "any one of claims 5 to 7" and insert --claim 5--.

Claim 12, line 1, delete "or claim 11".

Claim 13, line 1, delete "or claim 11".

Claim 14, line 1, delete "any one of claims 10 to 13" and insert --claim 10--.

Claim 15, line 1, delete "any one of claims 10 to 13" and insert --claim 10--.

Claim 17, line 1, delete "any one of claims 10 to 16" and insert --claim 10--.

Claim 18, line 1, delete "any one of claims 10 to 17" and insert --claim 10--.

Claim 19, line 1, delete "any one of claims 10 to 18" and insert --claim 10--.

Claim 20, line 1, delete "any one of claims 10 to 18" and insert --claim 10--.

Claim 22, line 1, delete "any one of claims 10 to 21" and insert --claim 10--.

Claim 23, line 1, delete "any one of claims 10 to 22" and insert --claim 10--.

Claim 24, line 1, delete "any one of claims 10 to 23" and insert --claim 10--.

Claim 25, line 1, delete "any one of claims 10 to 24" and insert --claim 10--.

Claim 29, line 1, delete "any one of claims 27 to 28" and insert --claim 27--.

Claim 30, line 1, delete "any one of claims 27 to 29" and insert --claim 27--.

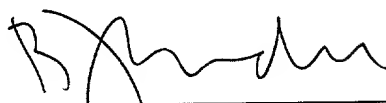
REMARKS

The claims have been amended to reduce the claims fees.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____



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Much research has been carried out on Ig molecules to determine how the variable domains are produced. It has been shown that each variable domain comprises a number of areas of relatively conserved sequence and three areas of hypervariable sequence. The three hypervariable areas are generally known as complementarity determining regions (CDRs).

Crystallographic studies have shown that in each variable domain of an Ig molecule the CDRs are supported on framework areas formed by the areas of conserved sequences. The three CDRs are brought together by the framework areas and, together with the CDRs on the other chain, form a pocket in which the target molecule is received.

15

Since the advent of recombinant DNA technology, there has been much interest in the use of such technology to clone and express Ig molecules and derivatives thereof. This interest is reflected in the numbers of patent applications and other publications on the subject.

The earliest work on the cloning and expression of full Igs in the patent literature is EP-A-0 120 694 (Boss). The Boss application also relates to the cloning and expression of chimeric antibodies. Chimeric antibodies are Ig-type molecules in which the variable domains from one Ig are fused to constant domains from another Ig. Usually, the variable domains are derived from an Ig from one species (often a mouse Ig) and the constant domains are derived from an Ig from a different species (often a human Ig).

A later European patent application, EP-A-0 125 023 (Genentech), relates to much the same subject as the Boss application, but also relates to the production by recombinant DNA technology of other variations of Ig-type molecules.

EP-A-0 194 276 (Neuberger) discloses not only chimeric antibodies of the type disclosed in the Boss application but also chimeric antibodies in which some or all of the constant domains have been replaced by non-Ig derived protein sequences. For instance, the heavy chain CH2 and CH3 domains may be replaced by protein sequences derived from an enzyme or a protein toxin.

EP-A-0 239 400 (Winter) discloses a different approach to the production of Ig molecules. In this approach, only the CDRs from a first type of Ig are grafted onto a second type of Ig in place of its normal CDRs. The Ig molecule thus produced is predominantly of the second type, since the CDRs form a relatively small part of the whole Ig. However, since the CDRs are the parts which define the specificity of the Ig, the Ig molecule thus produced has its specificity derived from the first Ig.

Hereinafter, chimeric antibodies, CDR-grafted Igs, the altered antibodies described by Genentech, and fragments, of such Igs such as F(ab')₂ and Fv fragments are referred to herein as modified antibodies.

One of the main reasons for all the activity in the Ig field using recombinant DNA technology is the desire to use Igs in therapy. It is well known that, using the hybridoma technique developed by Kohler and Milstein, it is possible to produce monoclonal antibodies (MAbs) of almost any specificity. Thus, MAbs directed against cancer antigens have been produced. It is envisaged that these MAbs could be covalently attached or fused to toxins to provide "magic bullets" for use in cancer therapy. MAbs directed against normal tissue or cell surface antigens have also been produced. Labels can be attached to these so that they can be used for in vivo imaging.

The major obstacle to the use of such MAbs in therapy or in vivo diagnosis is that the vast majority of MAbs which are

35 For light chains, the situation is similar, except that for light chains there is no diversity region. Thus light chain variable domains are encoded by an "unrearranged" VL gene and a JL gene. There are two types of light chains, kappa

(κ) or lambda (λ), which are built respectively from unrearranged $V\kappa$ genes and $J\kappa$ segments, and from unrearranged $V\lambda$ genes and $J\lambda$ segments.

- 5 Previous work has shown that it is necessary to have two variable domains in association together for efficient binding. For example, the associated heavy and light chain variable domains were shown to contain the antigen binding site [1]. This assumption is borne out by X-ray
10 crystallographic studies of crystallised antibody/antigen complexes [2-6] which show that both the heavy and light chains of the antibody's variable domains contact the antigen. The expectation that association of heavy and light chain variable domains is necessary for efficient antigen
15 binding underlies work to co-secrete these domains from bacteria [1], and to link the domains together by a short section of polypeptide as in the single chain antibodies [8, 9].
- 20 Binding of isolated heavy and light chains had also been detected. However the evidence suggested strongly that this was a property of heavy or light chain dimers. Early work, mainly with polyclonal antibodies, in which antibody heavy and light chains had been separated under denaturing
25 conditions [10] suggested that isolated antibody heavy chains could bind to protein antigens [11] or hapten [12]. The binding of protein antigen was not characterised, but the hapten-binding affinity of the heavy chain fragments was reduced by two orders of magnitude [12] and the number of
30 hapten molecules binding were variously estimated as 0.14 or 0.37 [13] or 0.26 [14] per isolated heavy chain. Furthermore binding of haptens was shown to be a property of dimeric heavy or dimeric light chains [14]. Indeed light chain dimers have been crystallised. It has been shown that in
35 light chain dimers the two chains form a cavity which is able to bind to a single molecule of hapten [15].

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This confirms the assumption that, in order to obtain efficient binding, it is necessary to have a dimer, and preferably a heavy chain/light chain dimer, containing the respective variable domains. This assumption also underlies the teaching of the patent references cited above, wherein the intention is always to produce dimeric, and preferably heavy/light chain dimeric, molecules.

It has now been discovered, contrary to expectations, that isolated Ig heavy chain variable domains can bind to antigen in a 1:1 ratio and with binding constants of equivalent magnitude to those of complete antibody molecules. In view of what was known up until now and in view of the assumptions made by those skilled in the art, this is highly surprising.

Therefore, according to a first aspect of the present invention, there is provided a single domain ligand consisting at least part of the variable domain of one chain of a molecule from the Ig.superfamily.

Preferably, the ligand consists of the variable domain of an Ig light, or, most preferably, heavy chain.

The ligand may be produced by any known technique, for instance by controlled cleavage of Ig superfamily molecules or by peptide synthesis. However, preferably the ligand is produced by recombinant DNA technology. For instance, the gene encoding the rearranged gene for a heavy chain variable domain may be produced, for instance by cloning or gene synthesis, and placed into a suitable expression vector. The expression vector is then used to transform a compatible host cell which is then cultured to allow the ligand to be expressed and, preferably, secreted.

If desired, the gene for the ligand can be mutated to improve the properties of the expressed domain, for example to increase the yields of expression or the solubility of

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the ligand, to enable the ligand to bind better, or to introduce a second site for covalent attachment (by introducing chemically reactive residues such as cysteine and histidine) or non-covalent binding of other molecules.

- 5 In particular it would be desirable to introduce a second site for binding to serum components, to prolong the residence time of the domains in the serum; or for binding to molecules with effector functions, such as components of complement, or receptors on the surfaces of cells.

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Thus, hydrophobic residues which would normally be at the interface of the heavy chain variable domain with the light chain variable domain could be mutated to more hydrophilic residues to improve solubility; residues in the CDR loops
15 could be mutated to improve antigen binding; residues on the other loops or parts of the β -sheet could be mutated to introduce new binding activities. Mutations could include single point mutations, multiple point mutations or more extensive changes and could be introduced by any of a
20 variety of recombinant DNA methods, for example gene synthesis, site directed mutagenesis or the polymerase chain reaction.

Since the ligands of the present invention have equivalent
25 binding affinity to that of complete Ig molecules, the ligands can be used in many of the ways as are Ig molecules or fragments. For example, Ig molecules have been used in therapy (such as in treating cancer, bacterial and viral diseases), in diagnosis (such as pregnancy testing), in
30 vaccination (such as in producing anti-idiotypic antibodies which mimic antigens), in modulation of activities of hormones or growth factors, in detection, in biosensors and in catalysis.

35 It is envisaged that the small size of the ligands of the present invention may confer some advantages over complete antibodies, for example, in neutralising the activity of low molecular weight drugs (such as digoxin) and allowing their

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filtration from the kidneys with drug attached; in penetrating tissues and tumours; in neutralising viruses by binding to small conserved regions on the surfaces of viruses such as the "canyon" sites of viruses [16]; in high resolution epitope mapping of proteins; and in vaccination by ligands which mimic antigens.

The present invention also provides receptors comprising a ligand according to the first aspect of the invention linked to one or more of an effector molecule, a label, a surface, or one or more other ligands having the same or different specificity.

A receptor comprising a ligand linked to an effector molecule may be of use in therapy. The effector molecule may be a toxin, such as ricin or pseudomonas exotoxin, an enzyme which is able to activate a prodrug, a binding partner or a radio-isotope. The radio-isotope may be directly linked to the ligand or may be attached thereto by a chelating structure which is directly linked to the ligand. Such ligands with attached isotopes are much smaller than those based on Fv fragments, and could penetrate tissues and access tumours more readily.

A receptor comprising a ligand linked to a label may be of use in diagnosis. The label may be a heavy metal atom or a radio-isotope, in which case the receptor can be used for in vivo imaging using X-ray or other scanning apparatus. The metal atom or radio-isotope may be attached to the ligand either directly or via a chelating structure directly linked to the ligand. For in vitro diagnostic testing, the label may be a heavy metal atom, a radio-isotope, an enzyme, a fluorescent or coloured molecule or a protein or peptide tag which can be detected by an antibody, an antibody fragment or another protein. Such receptors would be used in any of the known diagnostic tests, such as ELISA or fluorescence-linked assays.

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If desired, such receptors with multiple ligands could include effector molecules or labels so that they can be used in therapy or diagnosis as described above.

5 The ligand may be linked to the other part of the receptor by any suitable means, for instance by covalent or non-covalent chemical linkages. However, where the receptor comprises a ligand and another protein molecule, it is preferred that they are produced by recombinant DNA
10 technology as a fusion product. If necessary, a linker peptide sequence can be placed between the ligand and the other protein molecule to provide flexibility.

The basic techniques for manipulating Ig molecules by recombinant DNA technology are described in the patent references cited above. These may be adapted in order to allow for the production of ligands and receptors according to the invention by means of recombinant DNA technology.

20 Preferably, where the ligand is to be used for in vivo diagnosis or therapy in humans, it is humanised, for instance by CDR replacement as described in EP-A-0 239 400.

In order to obtain a DNA sequence encoding a ligand, it is generally necessary firstly to produce a hybridoma which secretes an appropriate MAb. This can be a very time consuming method. Once an immunised animal has been produced, it is necessary to fuse separated spleen cells with a suitable myeloma cell line, grow up the cell lines thus produced, select appropriate lines, reclone the selected lines and reselect. This can take some long time. This problem also applies to the production of modified Igs.

A further problem with the production of ligands, and also
35 receptors according to the invention and modified Igs, by
recombinant DNA technology is the cloning of the variable
domain encoding sequences from the hybridoma which produces
the MAb from which the specificity is to be derived. This

can be a relatively long method involving the production of a suitable probe, construction of a clone library from cDNA or genomic DNA, extensive probing of the clone library, and manipulation of any isolated clones to enable the cloning
5 into a suitable expression vector. Due to the inherent variability of the DNA sequences encoding Ig variable domains, it has not previously been possible to avoid such time consuming work. It is therefore a further aim of the present invention to provide a method which enables
10 substantially any sequence encoding an Ig superfamily molecule variable domain (ligand) to be cloned in a reasonable period of time.

According to another aspect of the present invention
15 therefore, there is provided a method of cloning a sequence (the target sequence) which encodes at least part of the variable domain of an Ig superfamily molecule, which method comprises:

- (a) providing a sample of double stranded (ds) nucleic
20 acid which contains the target sequence;
- (b) denaturing the sample so as to separate the two strands;
- (c) annealing to the sample a forward and a back oligonucleotide primer, the forward primer being specific
25 for a sequence at or adjacent the 3' end of the sense strand of the target sequence, the back primer being specific for a sequence at or adjacent the 3' end of the antisense strand of the target sequence, under conditions which allow the primers to hybridise to the nucleic acid at or adjacent the
30 target sequence;
- (d) treating the annealed sample with a DNA polymerase enzyme in the presence of deoxynucleoside triphosphates under conditions which cause primer extension to take place; and
- 35 (e) denaturing the sample under conditions such that the extended primers become separated from the target sequence.

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Preferably, the method of the present invention further includes the step (f) of repeating steps (c) to (e) on the denatured mixture a plurality of times.

5 Preferably, the method of the present invention is used to clone complete variable domains from Ig molecules, most preferably from Ig heavy chains. In the most preferred instance, the method will produce a DNA sequence encoding a ligand according to the present invention.

10

In step (c) recited above, the forward primer becomes annealed to the sense strand of the target sequence at or adjacent the 3' end of the strand. In a similar manner, the back primer becomes annealed to the antisense strand of the target sequence at or adjacent the 3' end of the strand. Thus, the forward primer anneals at or adjacent the region of the ds nucleic acid which encodes the C terminal end of the variable region or domain. Similarly, the back primer anneals at or adjacent the region of the ds nucleic acid which encodes the N-terminal end of the variable domain.

In step (d), nucleotides are added onto the 3' end of the forward and back primers in accordance with the sequence of the strand to which they are annealed. Primer extension will continue in this manner until stopped by the beginning of the denaturing step (e). It must therefore be ensured that step (d) is carried out for a long enough time to ensure that the primers are extended so that the extended strands totally overlap one another.

30

In step (e), the extended primers are separated from the ds nucleic acid. The ds nucleic acid can then serve again as a substrate to which further primers can anneal. Moreover, the extended primers themselves have the necessary complementary sequences to enable the primers to anneal thereto.

During further cycles, if step (f) is used, the amount of extended primers will increase exponentially so that at the end of the cycles there will be a large quantity of cDNA having sequences complementary to the sense and antisense
5 strands of the target sequence. Thus, the method of the present invention will result in the accumulation of a large quantity of cDNA which can form ds cDNA encoding at least part of the variable domain.

10 As will be apparent to the skilled person, some of the steps in the method may be carried out simultaneously or sequentially as desired.

The forward and back primers may be provided as isolated
15 oligonucleotides, in which case only two oligonucleotides will be used. However, alternatively the forward and back primers may each be supplied as a mixture of closely related oligonucleotides. For instance, it may be found that at a particular point in the sequence to which the primer is to
20 anneal, there is the possibility of nucleotide variation. In this case a primer may be used for each possible nucleotide variation. Furthermore it may be possible to use two or more sets of "nested" primers in the method to enhance the specific cloning of variable region genes.

25 The method described above is similar to the method described by Saiki et al. [17]. A similar method is also used in the methods described in EP-A-0 200 362. In both cases the method described is carried out using primers
30 which are known to anneal efficiently to the specified nucleotide sequence. In neither of these disclosures was it suggested that the method could be used to clone Ig parts of variable domain encoding sequences, where the target sequence contains inherently highly variable areas.

35 The ds nucleic acid sequence used in the method of the present invention may be derived from mRNA. For instance, RNA may be isolated in known manner from a cell or cell line

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which is known to produce Igs. mRNA may be separated from other RNA by oligo-dT chromatography. A complementary strand of cDNA may then be synthesised on the mRNA template, using reverse transcriptase and a suitable primer, to yield
5 an RNA/DNA heteroduplex. A second strand of DNA can be made in one of several ways, for example, by priming with RNA fragments of the mRNA strand (made by incubating RNA/DNA heteroduplex with RNase H) and using DNA polymerase, or by priming with a synthetic oligodeoxynucleotide primer which
10 anneals to the 3' end of the first strand and using DNA polymerase. It has been found that the method of the present invention can be carried out using ds cDNA prepared in this way.

15 When making such ds cDNA, it is possible to use a forward primer which anneals to a sequence in the CH1 domain (for a heavy chain variable domain) or the C α or C κ domain (for a light chain variable domain). These will be located in close enough proximity to the target sequence to allow the
20 sequence to be cloned.

The back primer may be one which anneals to a sequence at the N-terminal end of the VH1, V κ or V λ domain. The back primer may consist of a plurality of primers having a
25 variety of sequences designed to be complementary to the various families of VH1, V κ or V λ sequences known. Alternatively the back primer may be a single primer having a consensus sequence derived from all the families of variable region genes.

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Surprisingly, it has been found that the method of the present invention can be carried out using genomic DNA. If genomic DNA is used, there is a very large amount of DNA present, including actual coding sequences, introns and
35 untranslated sequences between genes. Thus, there is considerable scope for non-specific annealing under the conditions used. However, it has surprisingly been found that there is very little non-specific annealing. It is

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therefore unexpected that it has proved possible to clone the genes of Ig-variable domains from genomic DNA.

Under some circumstances the use of genomic DNA may prove
5 advantageous compared with use of mRNA, as the mRNA is
readily degraded, and especially difficult to prepare from
clinical samples of human tissue.

Thus, in accordance with an aspect of the present invention,
10 the ds nucleic acid used in step (a) is genomic DNA.

When using genomic DNA as the ds nucleic acid source, it will not be possible to use as the forward primer an oligonucleotide having a sequence complementary to a sequence in a constant domain. This is because, in genomic DNA, the constant domain genes are generally separated from the variable domain genes by a considerable number of base pairs. Thus, the site of annealing would be too remote from the sequence to be cloned.

20 It should be noted that the method of the present invention can be used to clone both rearranged and unrearranged variable domain sequences from genomic DNA. It is known that in germ line genomic DNA the three genes, encoding the
25 VH, DH and JH respectively, are separated from one another by considerable numbers of base pairs. On maturation of the immune response, these genes are rearranged so that the VH, DH and JH genes are fused together to provide the gene encoding the whole variable domain (see Figure 1). By using
30 a forward primer specific for a sequence at or adjacent the 3' end of the sense strand of the genomic "unrearranged" VH gene, it is possible to clone the "unrearranged" VH gene alone, without also cloning the DH and JH genes. This can be of use in that it will then be possible to fuse the VH
35 gene onto pre-cloned or synthetic DH and DH genes. In this way, rearrangement of the variable domain genes can be carried out in vitro.

[illegible]

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Advantageously, however, each primer includes a sequence including a restriction enzyme recognition site. The

sequence recognised by the restriction enzyme need not be in the part of the primer which anneals to the ds nucleic acid, but may be provided as an extension which does not anneal. The use of primers with restriction sites has the advantage

5 that the DNA can be cut with at least one restriction enzyme which leaves 3' or 5' overhanging nucleotides. Such DNA is more readily cloned into the corresponding sites on the vectors than blunt end fragments taken directly from the method. The ds cDNA produced at the end of the cycles will

10 thus be readily insertable into a cloning vector by use of the appropriate restriction enzymes. Preferably the choice of restriction sites is such that the ds cDNA is cloned directly into an expression vector, such that the ligand encoded by the gene is expressed. In this case the

15 restriction site is preferably located in the sequence which is annealed to the ds nucleic acid.

Since the primers may not have a sequence exactly complementary to the target sequence to which it is to be

20 annealed, for instance because of nucleotide variations or because of the introduction of a restriction enzyme recognition site, it may be necessary to adjust the conditions in the annealing mixture to enable the primers to anneal to the ds nucleic acid. This is well within the

25 competence of the person skilled in the art and needs no further explanation.

In step (d), any DNA polymerase may be used. Such polymerases are known in the art and are available

30 commercially. The conditions to be used with each polymerase are well known and require no further explanation here. The polymerase reaction will need to be carried out in the presence of the four nucleoside triphosphates. These and the polymerase enzyme may already be present in the

35 sample or may be provided afresh for each cycle.

The denaturing step (e) may be carried out, for instance, by heating the sample, by use of chaotropic agents, such as

urea or guanidine, or by the use of changes in ionic strength or pH. Preferably, denaturing is carried out by heating since this is readily reversible. Where heating is used to carry out the denaturing, it will be usual to use a thermostable DNA polymerase, such as Taq polymerase, since this will not need replenishing at each cycle.

If heating is used to control the method, a suitable cycle of heating comprises denaturation at about 95°C for about 1 minute, annealing at from 30°C to 65°C for about 1 minute and primer extension at about 75°C for about 2 minutes. To ensure that elongation and renaturation is complete, the mixture after the final cycle is preferably held at about 60°C for about 5 minutes.

15 The product ds cDNA may be separated from the mixture for instance by gel electrophoresis using agarose gels. However, if desired, the ds cDNA may be used in unpurified form and inserted directly into a suitable cloning or expression
20 vector by conventional methods. This will be particularly easy to accomplish if the primers include restriction enzyme recognition sequences.

The method of the present invention may be used to make variations in the sequences encoding the variable domains. For example this may be achieved by using a mixture of related oligonucleotide primers as at least one of the primers. Preferably the primers are particularly variable in the middle of the primer and relatively conserved at the 5' and 3' ends. Preferably the ends of the primers are complementary to the framework regions of the variable domain, and the variable region in the middle of the primer covers all or part of a CDR. Preferably a forward primer is used in the area which forms the third CDR. If the method is carried out using such a mixture of oligonucleotides, the product will be a mixture of variable domain encoding sequences. Moreover, variations in the sequence may be introduced by incorporating some mutagenic nucleotide

5 error rate of the DNA polymerase are amplified, particularly when using high concentrations of nucleoside triphosphates.

10 variable domain encoding sequences directly from mRNA or genomic DNA. This in turn will facilitate the production of modified Ig-type molecules by any of the prior art methods referred to above. Further, target genes can be cloned from tissue samples containing antibody producing cells, and the
15 genes can be sequenced. By doing this, it will be possible to look directly at the immune repertoire of a patient. This "fingerprinting" of a patient's immune repertoire could be of use in diagnosis, for instance of auto-immune diseases.

20 In the method for amplifying the amount of a gene encoding
a variable domain, a single set of primers is used in
several cycles of copying via the polymerase chain reaction.
As a less preferred alternative, there is provided a second
method which comprises steps (a) to (d) as above, which
25 further includes the steps of:

(g) treating the sample of ds cDNA with traces of DNase in the presence of DNA polymerase I to allow nick translation of the DNA; and

(h) cloning the ds cDNA into a vector.

30 If desired, the second method may further include the steps
of:

(i) digesting the DNA of recombinant plasmids to release DNA fragments containing genes encoding variable domains;

35 and

(j) treating the fragments in a further set of steps (c) to (h).

Preferably the fragments are separated from the vector and from other fragments of the incorrect size by gel electrophoresis.

5 The steps (a) to (d) then (g) to (h) can be followed once, but preferably the entire cycle (c) to (d) and (g) to (j) is repeated at least once. In this way a priming step, in which the genes are specifically copied, is followed by a cloning step, in which the amount of genes is increased.

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In step (a) the ds cDNA is derived from mRNA. For Ig derived variable domains, the mRNA is preferably be isolated from lymphocytes which have been stimulated to enhance production of mRNA.

15

In each step (c) the set of primers are preferably different from the previous step (c), so as to enhance the specificity of copying. Thus the sets of primers form a nested set. For example, for cloning of Ig heavy chain variable domains, the
20 first set of primers may be located within the signal sequence and constant region, as described by Larrick et al., [18], and the second set of primers entirely within the variable region, as described by Orlandi et al., [19]. Preferably the primers of step (c) include restriction sites
25 to facilitate subsequent cloning. In the last cycle the set of primers used in step (c) should preferably include restriction sites for introduction into expression vectors. In step (g) possible mismatches between the primers and the template strands are corrected by "nick translation". In
30 step (h), the ds cDNA is preferably cleaved with restriction enzymes at sites introduced into the primers to facilitate the cloning.

According to another aspect of the present invention the
35 product ds cDNA is cloned directly into an expression vector. The host may be prokaryotic or eukaryotic, but is preferably bacterial. Preferably the choice of restriction sites in the primers and in the vector, and other features

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Single ligands could be expressed alone or in combination with a complementary variable domain. For example, a heavy chain variable domain can be expressed either as an individual domain or, if it is expressed with a complementary light chain variable domain, as an antigen binding site. Preferably the two partners would be expressed in the same cell, or secreted from the same cell, and the proteins allowed to associate non-covalently to form an Fv fragment. Thus the two genes encoding the complementary partners can be placed in tandem and expressed from a single vector, the vector including two sets of restriction sites.

Preferably the genes are introduced sequentially: for example the heavy chain variable domain can be cloned first and then the light chain variable domain. Alternatively the two genes are introduced into the vector in a single step, for example by using the polymerase chain reaction to paste together each gene with any necessary intervening sequence, as essentially described by Yon and Fried [29]. The two partners could be also expressed as a linked protein to produce a single chain Fv fragment, using similar vectors to those described above. As a further alternative the two genes may be placed in two different vectors, for example in which one vector is a phage vector and the other is a plasmid vector.

Moreover, the cloned ds cDNA may be inserted into an expression vector already containing sequences encoding one or more constant domains to allow the vector to express Ig-type chains. The expression of Fab fragments, for example, would have the advantage over Fv fragments that the heavy and light chains would tend to associate through the constant domains in addition to the variable domains. The final expression product may be any of the modified Ig-type molecules referred to above.

The cloned sequence may also be inserted into an expression vector so that it can be expressed as a fusion protein. The variable domain encoding sequence may be linked directly or via a linker sequence to a DNA sequence encoding any protein effector molecule, such as a toxin, enzyme, label or another ligand. The variable domain sequences may also be linked to proteins on the outer side of bacteria or phage. Thus, the method of this aspect of the invention may be used to produce receptors according to the invention.

According to another aspect of the invention, the cloning of ds cDNA directly for expression permits the rapid construction of expression libraries which can be screened

for binding activities. For Ig heavy and light chain variable genes, the ds cDNA may comprise variable genes isolated as complete rearranged genes from the animal, or variable genes built from several different sources, for example a repertoire of unrearranged VH genes combined with a synthetic repertoire of DH and JH genes. Preferably repertoires of genes encoding Ig heavy chain variable domains are prepared from lymphocytes of animals immunised with an antigen.

10

The screening method may take a range of formats well known in the art. For example Ig heavy chain variable domains secreted from bacteria may be screened by binding to antigen on a solid phase, and detecting the captured domains by antibodies. Thus the domains may be screened by growing the bacteria in liquid culture and binding to antigen coated on the surface of ELISA plates. However, preferably bacterial colonies (or phage plaques) which secrete ligands (or modified ligands, or ligand fusions with proteins) are screened for antigen binding on membranes. Either the ligands are bound directly to the membranes (and for example detected with labelled antigen), or captured on antigen coated membranes (and detected with reagents specific for ligands). The use of membranes offers great convenience in screening many clones, and such techniques are well known in the art.

The screening method may also be greatly facilitated by making protein fusions with the ligands, for example by introducing a peptide tag which is recognised by an antibody at the N-terminal or C-terminal end of the ligand, or joining the ligand to an enzyme which catalyses the conversion of a colourless substrate to a coloured product. In the latter case, the binding of antigen may be detected simply by adding substrate. Alternatively, for ligands expressed and folded correctly inside eukaryotic cells, joining of the ligand and a domain of a transcriptional activator such as the GAL4 protein of yeast, and joining of

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antigen to the other domain of the GAL4 protein, could form the basis for screening binding activities, as described by Fields and Song [21].

- 5 The preparation of proteins, or even cells with multiple copies of the ligands, may improve the avidity of the ligand for immobilised antigen, and hence the sensitivity of the screening method. For example, the ligand may be joined to a protein subunit of a multimeric protein, to a phage coat
10 protein or to an outer membrane protein of *E. coli* such as ompA or lamB. Such fusions to phage or bacterial proteins also offers possibilities of selecting bacteria displaying ligands with antigen binding activities. For example such bacteria may be precipitated with antigen bound to a solid
15 support, or may be subjected to affinity chromatography, or may be bound to larger cells or particles which have been coated with antigen and sorted using a fluorescence activated cell sorter (FACS). The proteins or peptides fused to the ligands are preferably encoded by the vector,
20 such that cloning of the ds cDNA repertoire creates the fusion product.

- In addition to screening for binding activities of single ligands, it may be necessary to screen for binding or
25 catalytic activities of associated ligands, for example, the associated Ig heavy and light chain variable domains. For example, repertoires of heavy and light chain variable genes may be cloned such that two domains are expressed together. Only some of the pairs of domains may associate, and only
30 some of these associated pairs may bind to antigen. The repertoires of heavy and light chain variable domains could be cloned such that each domain is paired at random. This approach may be most suitable for isolation of associated domains in which the presence of both partners is required
35 to form a cleft. Alternatively, to allow the binding of hapten. Alternatively, since the repertoires of light chain sequences are less diverse than those of heavy chains, a small repertoire of light chain variable domains, for

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Alternatively the heavy and light chain variable domains are covalently linked together with a peptide, as in the single chain antibodies, or peptide sequences attached, preferably at the C-terminal end which will associate through forming
5 cysteine bonds or through non-covalent interactions, such as the introduction of "leucine zipper" motifs. However, in order to isolate pairs of tightly associated variable domains, the Fv fragments are preferably used.

10 The construction of Fv fragments isolated from a repertoire of variable region genes offers a way of building complete antibodies, and an alternative to hybridoma technology. For example by attaching the variable domains to light or suitable heavy chain constant domains, as appropriate, and
15 expressing the assembled genes in mammalian cells, complete antibodies may be made and should possess natural effector functions, such as complement lysis. This route is particularly attractive for the construction of human monoclonal antibodies, as hybridoma technology has proved
20 difficult, and for example, although human peripheral blood lymphocytes can be immortalised with Epstein Barr virus, such hybridomas tend to secrete low affinity IgM antibodies.

Moreover, it is known that immunological mechanisms ensure
25 that lymphocytes do not generally secrete antibodies directed against host proteins. However it is desirable to make human antibodies directed against human proteins, for example to human cell surface markers to treat cancers, or to histocompatibility antigens to treat auto-immune
30 diseases. The construction of human antibodies built from the combinatorial repertoire of heavy and light chain variable domains may overcome this problem, as it will allow human antibodies to be built with specificities which would normally have been eliminated.

35

The method also offers a new way of making bispecific antibodies. Antibodies with dual specificity can be made by fusing two hybridomas of different specificities, so as to

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30 Thus the use of the method of the present invention will allow for the production of heavy chain variable domains with binding activities and variants of these domains. It allows for the production of monoclonal antibodies and bispecific antibodies, and will provide an alternative to
35 hybridoma technology. For instance, mouse splenic ds mRNA or genomic DNA may be obtained from a hyperimmunised mouse. This could be cloned using the method of the present invention and then the cloned ds DNA inserted into a

10

Figure 1 shows a schematic representation of the unrearranged and rearranged heavy and light chain variable genes and the location of the primers;

20

Figure 4 shows a schematic representation of the M13-VKPCR1 vector and a cloning scheme for light chain variable domains;

Figure 5 shows the sequence of the Ig variable region derived sequences in M13-VKPCR1;

35

Figure 7 shows a schematic representation of the pSV-gpt vector (also known as α -Lys 30) which contains a variable region cloned as a HindIII-BamHI fragment, which is excised on introducing the new variable region. The gene for human IgG1 has also been engineered to remove a BamHI site, such that the BamHI site in the vector is unique;

Figure 8 shows a schematic representation of the pSV-hygro vector (also known as α -Lys 17). It is derived from pSV gpt vector with the gene encoding mycophenolic acid replaced by a gene coding for hygromycin resistance. The construct
5 contains a variable gene cloned as a HindIII-BamHI fragment which is excised on introducing the new variable region. The gene for human C κ has also been engineered to remove a BamHI site, such that the BamHI site in the vector is unique;

10

Figure 9 shows the assembly of the mouse: human MB1 chimaeric antibody;

Figure 10 shows encoded amino acid sequences of 48 mouse
15 rearranged VH genes;

Figure 11 shows encoded amino acid sequences of human rearranged VH genes;

20 Figure 12 shows encoded amino acid sequences of unrearranged human VH genes;

Figure 13 shows the sequence of part of the plasmid pSW1: essentially the sequence of a pectate lyase leader linked to
25 VHLYS in pSW1 and cloned as an SphI-EcoRI fragment into pUC19 and the translation of the open reading frame encoding the pectate lyase leader-VHLYS polypeptide being shown;

Figure 14 shows the sequence of part of the plasmid pSW2: essentially the sequence of a pectate lyase leader linked to
30 VHLYS and to VKLYS, and cloned as an SphI-EcoRI-EcoRI fragment into pUC19 and the translation of open reading frames encoding the pectate lyase leader-VHLYS and pectate lyase leader-VKLYS polypeptides being shown;

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Figure 15 shows the sequence of part of the plasmid pSW1HPOLYMYC which is based on pSW1 and in which a polylinker sequence has replaced the variable domain of

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VHLYS, and acts as a cloning site for amplified VH genes, and a peptide tag is introduced at the C-terminal end;

Figure 16 shows the encoded amino acid sequences of two VH domains derived from mouse spleen and having lysozyme binding activity, and compared with the VH domain of the D1,3 antibody. The arrows mark the points of difference between the two VH domains;

10 Figure 17 shows the encoded amino acid sequence of a VH domain derived from human peripheral blood lymphocytes and having lysozyme binding activity;

Figure 18 shows a scheme for generating and cloning mutants of the VHLYS gene, which is compared with the scheme for cloning natural repertoires of VH genes;

Figure 19 shows the sequence of part of the vector pSW2HPOLY;

20

Figure 20 shows the sequence of part of the vector pSW3 which encodes the two linked VHLYS domains;

Figure 21 shows the sequence of the VHLYS domain and pelB leader sequence fused to the alkaline phosphatase gene;

25

Figure 22 shows the sequence of the vector pSW1VHLYS-VKPOLYMYC for expression of a repertoire of V κ light chain variable domains in association with the VHLYS domain; and

30

Figure 23 shows the sequence of VH domain which is secreted at high levels from *E. coli*. The differences with VHLYS domain are marked.

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PRIMERS

In the Examples described below, the following oligonucleotide primers, or mixed primers were used. Their locations are marked on Figure 1 and sequences are as follows:

VH1FOR 5' TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG 3';
 VH1FOR-2 5' TGAGGAGACGGTGACCGTGGTCCCTTGGCCCC 3';

10 Hu1VHFOR 5' CTTGGTGGAGGCTGAGGAGACGGTGACC 3';
 Hu2VHFOR 5' CTTGGTGGAGGCTGAGGAGACGGTGACC 3';
 Hu3VHFOR 5' CTTGGTGGATGCTGAGGAGACGGTGACC 3';
 Hu4VHFOR 5' CTTGGTGGATGCTGATGAGACGGTGACC 3';

15 MOJH1FOR 5' TGAGGAGACGGTGACCGTGGTCCCTGCGCCCCAG 3';
 MOJH2FOR 5' TGAGGAGACGGTGACCGTGGTGCCTTGGCCCCAG 3';
 MOJH3FOR 5' TGCAGAGACGGTGACCAGAGTCCCTTGGCCCCAG 3';
 MOJH4FOR 5' TGAGGAGACGGTGACCGAGGTTCCCTTGACCCCAG 3';

20 HUIJH1FOR 5' TGAGGAGACGGTGACCAGGGTGCCCTGGCCCCAG 3';
 HUIJH2FOR 5' TGAGGAGACGGTGACCAGGGTGCCACGGCCCCAG 3';
 HUIJH4FOR 5' TGAGGAGACGGTGACCAGGGTTCCTTGGCCCCAG 3';

25 VK1FOR 5' GTTAGATCTCCAGCTTGGTCCC 3';
 VK2FOR 5' CGTTAGATCTCCAGCTTGGTCCC 3';
 VK3FOR 5' CCGTTTCAGCTCGAGCTTGGTCCC 3';

MOJK1FOR 5' CGTTAGATCTCCAGCTTGGTGCC 3';
 30 MOJK3FOR 5' GGTTAGATCTCCAGTCTGGTCCC 3';
 MOJK4FOR 5' CGTTAGATCTCCAACCTTTGTCCC 3';

HUJK1FOR 5' CGTTAGATCTCCACCTTGGTCCC 3';
 HUJK3FOR 5' CGTTAGATCTCCACTTTGGTCCC 3';
 35 HUJK4FOR 5' CGTTAGATCTCCACCTTGGTCCC 3';
 HUJK5FOR 5' CGTTAGATCTCCAGTCGTGTCCC 3';

VH1BACK 5' AGGT(C/G)(C/A)A(G/A)CTGCAG(G/C)AGTC(T/A)GG 3';

Hu2VHIBACK: 5' CAGGTGCAGCTGCAGCAGTCTGG 3';
HuVHIIIBACK: 5' CAGGTGCAGCTGCAGGAGTCGGG 3';
Hu2VHIIIBACK: 5' GAGGTGCAGCTGCAGGAGTCTGG 3';
5 HuVHIVBACK: 5' CAGGTGCAGCTGCAGCAGTCTGG 3';

MOVHIBACK 5' AGGTGCAGCTGCAGGAGTCAG 3';
MOVHIIIBACK 5' AGGTCCAGCTGCAGCA(G/A)TCTGG 3';
MOVHIIIBACK 5' AGGTCCAACTGCAGCAGCCTGG 3';
10 MOVHIIIBACK 5' AGGTGAAGCTGCAGGAGTCTGG 3';

VK1BACK 5' GACATTCAGCTGACCCAGTCTCCA 3';
VK2BACK 5' GACATTGAGCTCACCCAGTCTCCA 3';

15 MOVKIIIBACK 5' GATGTTCACTGACCCAAACTCCA 3';
MOVKIIIBACK 5' GATATTCAGCTGACCCAGGATGAA 3';

HuHep1FOR 5' C(A/G)(C/G)TGAGCTCACTGTGTCTCTCGCACA 3';
HuOcta1BACK 5' CGTGAATATGCAAATAA 3';
20 HuOcta2BACK 5' AGTAGGAGACATGCAAAT 3'; and
HuOcta3BACK 5' CACCACCCACATGCAAAT 3';

VHMUT1 5' GGAGACGGTGACCGTGGTCCCTTGGCCCCAGTAGTCAAG
NNNNNNNNNNNNCTCTCTGGC 3' (where N is an
25 equimolar mixture of T, C, G and A)
M13 PRIMER 5' AACAGCTATGACCATG 3' (New England Biolabs
*1201)

EXAMPLE 1

Cloning of Mouse Rearranged Variable region genes from
hybridomas, assembly of genes encoding chimaeric antibodies
5 and the expression of antibodies from myeloma cells

VH1FOR is designed to anneal with the 3' end of the sense strand of any mouse heavy chain variable domain encoding sequence. It contains a BstEII recognition site. VK1FOR is
10 designed to anneal with the 3' end of the sense strand of any mouse kappa-type light chain variable domain encoding sequence and contains a BglII recognition site. VH1BACK is designed to anneal with the 3' end of the antisense strand of any mouse heavy chain variable domain and contains a PstI
15 recognition site. VK1BACK is designed to anneal with the 3' end of the antisense strand of any mouse kappa-type light chain variable domain encoding sequence and contains a PvuII recognition site.

20 In this Example five mouse hybridomas were used as a source of ds nucleic acid. The hybridomas produce monoclonal antibodies (MAbs) designated MBrl [23], BW431/26 [24], BW494/32 [25], BW250/183 [24,26] and BW704/152 [27]. MAb MBrl is particularly interesting in that it is known to be
25 specific for a saccharide epitope on a human mammary carcinoma line MCF-7 [28].

Cloning via mRNA

30 Each of the five hybridomas referred to above was grown up in roller bottles and about 5×10^8 cells of each hybridoma were used to isolate RNA. mRNA was separated from the isolated RNA using oligodT cellulose [29]. First strand cDNA was synthesised according to the procedure described by
35 Maniatis et al. [30] as set out below.

In order to clone the heavy chain variable domain encoding sequence, a 50 μ l reaction solution which contains 10 μ g

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Amplification from RNA/DNA hybrid

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The procedure for light chain amplification was exactly as described above, except that the VK1FOR and VK1BACK primers were used in place of the VH1FOR and VH1BACK primers respectively.

5

5 μ l of each sample of amplified cDNA was fractionated on 2% agarose gels by electrophoresis and stained with ethidium bromide. This showed that the amplified ds cDNA gave a major band of the expected size (about 330 bp). (However the band
10 for VK DNA of MB1 was very weak. It was therefore excised from the gel and reamplified in a second round.) Thus by this simple procedure, reasonable quantities of ds DNA encoding the light and heavy chain variable domains of the five MAbs were produced.

15

Heavy Chain Vector Construction

A BstEII recognition site was introduced into the vector M13-HuVHNP [31] by site directed mutagenesis [32,33] to
20 produce the 'vector M13-VHPCR1' (Figures 2 and 3).

Each amplified heavy chain variable domain encoding sequence was digested with the restriction enzymes PstI and BstEII. The fragments were phenol extracted, purified on 2% low
25 melting point agarose gels and force cloned into vector M13-VHPCR1 which had been digested with PstI and BstEII and purified on an 0.8% agarose gel. Clones containing the variable domain inserts were identified directly by sequencing [34] using primers based in the 3' non-coding
30 variable gene in the M13-VHPCR1 vector.

There is an internal PstI site in the heavy chain variable domain encoding sequences of BW431/26. This variable domain encoding sequence was therefore assembled in two steps. The
35 3' PstI-BstEII fragment was first cloned into M13-VHPCR1, followed in a second step by the 5' PstI fragment.

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Light Chain Vector Construction

Vector M13mp18 [35] was cut with PvuII and the vector backbone was blunt ligated to a synthetic HindIII-BamHI polylinker. Vector M13-HuVKLYS [36] was digested with HindIII and BamHI to isolate the HuVKLYS gene. This HindIII-BamHI fragment was then inserted into the HindIII-BamHI polylinker site to form a vector M13-VKPCR1 which lacks any PvuII sites in the vector backbone (Figures 4 and 5). This vector was prepared in E Coli JM110 [22] to avoid dam methylation at the BclI site.

Each amplified light chain variable domain encoding sequence was digested with PvuII and BglII. The fragments were phenol extracted, purified on 2% low melting point agarose gels and force cloned into vector M13-VKPCR1 which had been digested with PvuII and BclI, purified on an 0.8% agarose gel and treated with calf intestinal phosphatase. Clones containing the light chain variable region inserts were identified directly by sequencing [34] using primers based in the 3' non-coding region of the variable domain in the M13-VKPCR1 vector.

The nucleotide sequences of the MB1 heavy and light chain variable domains are shown in Figure 6 with part of the flanking regions of the M13-VHPCR1 and M13-VKPCR1 vectors.

Antibody Expression

The HindIII-BamHI fragment carrying the MB1 heavy chain variable domain encoding sequence in M13-VHPCR1 was recloned into a pSV-gpt vector with human $\gamma 1$ constant regions [37] (Figure 7). The MB1 light chain variable domain encoding sequence in M13-VKPCR1 was recloned as a HindIII-BamHI fragment into a pSV vector, pSV-hyg-HuCK with a hygromycin resistance marker and a human kappa constant domain (Figure 8). The assembly of the genes is summarised in Figure 9.

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The vectors thus produced were linearised with PvuI (in the case of the pSV-hygro vectors the PvuI digest is only partial) and cotransfected into the non-secreting mouse myeloma line NSO [38] by electroporation [39]. One day after cotransfection, cells were selected in 0.3 µg/ml mycophenolic acid (MPA) and after seven days in 1µg/ml MPA. After 14 days, four wells, each containing one or two major colonies, were screened by incorporation of ¹⁴C-lysine [40] and the secreted antibody detected after precipitation with protein-A Sepharose™ (Pharmacia) on SDS-PAGE [41]. The gels were stained, fixed, soaked in a fluorographic reagent, Amplify™ (Amersham), dried and autoradiographed on preflashed film at -70°C for 2 days.

Supernatant was also tested for binding to the mammary carcinoma line MCF-7 and the colon carcinoma line HT-29, essentially as described by Menard et al. [23], either by an indirect immunofluorescence assay on cell suspensions (using a fluorescein-labelled goat anti-human IgG (Amersham)) or by a solid phase RIA on monolayers of fixed cells (using ¹²⁵I-protein A (Amersham)).

It was found that one of the supernatants from the four wells contained secreted antibody. The chimeric antibody in the supernatant, like the parent mouse MB1 antibody, was found to bind to MCF-7 cells but not the HT-29 cells, thus showing that the specificity had been properly cloned and expressed.

Example 2Cloning of rearranged variable genes from genomic DNA of mouse spleen

5

Preparation of DNA from spleen.

The DNA from the mouse spleen was prepared in one of two ways (although other ways can be used).

10

Method 1. A mouse spleen was cut into two pieces and each piece was put into a standard Eppendorf tube with 200 μ l of PBS. The tip of a 1 ml glass pipette was closed and rounded in the blue flame of a Bunsen burner. The pipette was used to squash the spleen piece in each tube. The cells thus produced were transferred to a fresh Eppendorf tube and the method was repeated three times until the connective tissue of the spleen appeared white. Any connective tissue which has been transferred with the cells was removed using a drawn-out Pasteur pipette. The cells were then washed in PBS and distributed into four tubes.

The mouse spleen cells were then sedimented by a 2 minute spin in a Microcentaur centrifuge at low speed setting. All the supernatant was aspirated with a drawn out Pasteur pipette. If desired, at this point the cell sample can be frozen and stored at -20°C

To the cell sample (once thawed if it had been frozen) was added 500 μ l of water and 5 μ l of a 10% solution of NP-40, a non-ionic detergent. The tube was closed and a hole was punched in the lid. The tube was placed on a boiling water bath for 5 minutes to disrupt the cells and was then cooled on ice for 5 minutes. The tube was then spun for 2 minutes at high speed to remove cell debris.

The supernatant was transferred to a new tube and to this was added 125 μ l 5M NaCl and 30 μ l 1M MOPS adjusted to pH

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7.0. The DNA in the supernatant was absorbed on a Quiagen 5 tip and purified following the manufacturer's instructions for lambda DNA. After isopropanol precipitation, the DNA was resuspended in 500 μ l water.

5

Method 2. This method is based on the technique described in Maniatis et al. [30]. A mouse spleen was cut into very fine pieces and put into a 2 ml glass homogeniser. The cells were then freed from the tissue by several slow up and down
10 strokes with the piston. The cell suspension was made in 500 μ l phosphate buffered saline (PBS) and transferred to an Eppendorf tube. The cells were then spun for 2 min at low speed in a Microcentaur centrifuge. This results in a visible separation of white and red cells. The white cells,
15 sedimenting slower, form a layer on top of the red cells. The supernatant was carefully removed and spun to ensure that all the white cells had sedimented. The layer of white cells was resuspended in two portions of 500 μ l PBS and transferred to another tube.

20

The white cells were precipitated by spinning in the Microcentaur centrifuge at low speed for one minute. The cells were washed a further two times with 500 μ l PBS, and were finally resuspended in 200 μ l PBS. The white cells were
25 added to 2.5 ml 25 mM EDTA and 10 mM Tris.Cl, pH 7.4, and vortexed slowly. While vortexing 25 μ l 20% SDS was added. The cells lysed immediately and the solution became viscous and clear. 100 μ l of 20 mg/ml proteinase K was added and incubated one to three hours at 50°C.

30

The sample was extracted with an equal volume of phenol and the same volume of chloroform, and vortexed. After centrifuging, the aqueous phase was removed and 1/10 volume 3M ammonium acetate was added. This was overlaid with three
35 volumes of cold ethanol and the tube rocked carefully until the DNA strands became visible. The DNA was spooled out with a Pasteur pipette, the ethanol allowed to drip off, and the DNA transferred to 1 ml of 10 mM Tris.Cl pH 7.4, 0.1 mM EDTA

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in an Eppendorf tube. The DNA was allowed to dissolve in the cold overnight on a roller.

Amplification from genomic DNA.

5

The DNA solution was diluted 1/10 in water and boiled for 5 min prior to using the polymerase chain reaction (PCR). For each PCR reaction, typically 50-200 ng of DNA were used.

- 10 The heavy and light chain variable domain encoding sequences in the genomic DNA isolated from the human PBL or the mouse spleen cells was then amplified and cloned using the general protocol described in the first two paragraphs of the section headed "Amplification from RNA/DNA Hybrid" in
- 15 Example 1, except that during the annealing part of each cycle, the temperature was held at 65°C and that 30 cycles were used. Furthermore, to minimise the annealing between the 3' ends of the two primers, the sample was first heated to 95°C, then annealed at 65°C, and only then was the Taq
- 20 polymerase added. At the end of the 30 cycles, the reaction mixture was held at 60°C for five minutes to ensure that complete elongation and renaturation of the amplified fragments had taken place.

- 25 The primers used to amplify the mouse spleen genomic DNA were VH1FOR and VH1BACK, for the heavy chain variable domain and VK2FOR and VK1BACK, for the light chain variable domain. (VK2FOR only differs from VK1FOR in that it has an extra C residue on the 5' end.)

30

- Other sets of primers, designed to optimise annealing with different families of mouse VH and V κ genes were devised and used in mixtures with the primers above. For example, mixtures of VK1FOR, MOJK1FOR, MOJK3FOR and MOJK4FOR were
- 35 used as forward primers and mixtures of VK1BACK, MOVKIIABACK and MOVKIIIBACK as back primers for amplification of V κ genes. Likewise mixtures of VH1FOR, MOJH1FOR, MOJH2FOR, MOJH3FOR and MOJH4FOR were used as forward primers and

mixtures of VH1BACK, MOVH1BACK, MOVH1IABACK, MOVH1IBBACK, MOVH1IIBACK were used as backward primers for amplification of VH genes.

- 5 All these heavy chain FOR primers referred to above contain a BstEII site and all the BACK primers referred to above contain a PstI site. These light chain FOR and BACK primers referred to above all contain BglII and PvuII sites respectively. Light chain primers (VK3FOR and VK2BACK) were
10 also devised which utilised different restriction sites, SacI and XhoI.

Typically all these primers yielded amplified DNA of the correct size on gel electrophoresis, although other bands
15 were also present. However, a problem was identified in which the 5' and 3' ends of the forward and backward primers for the VH genes were partially complementary, and this could yield a major band of "primer-dimer" in which the two oligonucleotides prime on each other. For this reason an
20 improved forward primer, VH1FOR-2 was devised in which the two 3' nucleotides were removed from VH1FOR.

Thus, the preferred amplification conditions for mouse VH genes are as follows: the sample was made in a volume of 50
25 -100 μ l, 50-100 ng of DNA, VH1FOR-2 and VH1BACK primers (25 pmole of each), 250 μ M of each deoxynucleotide triphosphate, 10 mM Tris.HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, and 100 μ g/ml gelatine. The sample was overlaid with paraffin oil, heated to 95° C for 2 min, 65° C for 2 min, and then to
30 72° C: taq polymerase was added after the sample had reached the elongation temperature and the reaction continued for 2 min at 72° C. The sample was subjected to a further 29 rounds of temperature cycling using the Techne PHC-1 programmable heating block.

35

The preferred amplification conditions for mouse Vk genes from genomic DNA are as follows: the sample treated as above except with Vk primers, for example VK3FOR and VK2BACK, and

The conditions which were devised for genomic DNA are also
5 suitable for amplification from the cDNA derived from mRNA
from mouse spleen or mouse hybridoma.

10 The reaction mixture was then extracted twice with 40 μ l of water-saturated diethyl ether. This was followed by a standard phenol extraction and ethanol precipitation as described in Example 1. The DNA pellet was then dissolved in 100 μ l 10 mM Tris.Cl, 0.1 mM EDTA.

The heavy chain variable genes isolated as above from a mouse hyperimmunised with lysozyme were cloned into M13VHPCR1 vector and sequenced. The complete sequences of 48 VH gene clones were determined (Figure 10). All but two of the mouse VH gene families were represented, with frequencies of: VA (1), IIIC (1), IIIB (8), IIIA (3), IIB (17), IIA (2), IB (12), IA (4). In 30 clones, the D segments could be assigned to families SP2 (14), FL16 (11) and Q52 (5), and in 38 clones the JH minigenes to families JH1 (3), JH2 (7), JH3 (14) and JH4 (14). The different sequences of CDR3 marked out each of the 48 clones as unique. Nine pseudogenes and 16 unproductive rearrangements were identified. Of the clones sequenced, 27 have open reading frames.

Thus the method is capable of generating a diverse repertoire of heavy chain variable genes from mouse spleen DNA.

5 Example 3

Cloning of rearranged variable genes from mRNA from human peripheral blood lymphocytes

10 Preparation of mRNA.

Human peripheral blood lymphocytes were purified and mRNA prepared directly (Method 1), or mRNA was prepared after addition of Epstein Barr virus (Method 2).

15

Method 1. 20 ml of heparinised human blood from a healthy volunteer was diluted with an equal volume of phosphate buffered saline (PBS) and distributed equally into 50 ml Falcon tubes. The blood was then underlayered with 15ml

20 Ficoll Hypaque (Pharmacia 10-A-001-07). To separate the lymphocytes from the red blood cells, the tubes were spun for 10 minutes at 1800 rpm at room temperature in an IEC Centra 3E table centrifuge. The peripheral blood lymphocytes (PBL) were then collected from the interphase by
25 aspiration with a Pasteur pipette. The cells were diluted with an equal volume of PBS and spun again at 1500 rpm for 15 minutes. The supernatant was aspirated, the cell pellet was resuspended in 1 ml PBS and the cells were distributed into two Eppendorf tubes.

30

Method 2. 40 ml human blood from a patient with HIV in the pre-AIDS condition was layered on Ficoll to separate the white cells (see Method 1 above). The white cells were then incubated in tissue culture medium for 4-5 days. On day 3,
35 they were infected with Epstein Barr virus. The cells were pelleted (approx 2×10^7 cells) and washed in PBS.

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The cells were pelleted again and lysed with 7 ml 5M guanidine isothiocyanate, 50 mM Tris, 10 mM EDTA, 0.1 mM dithiothreitol. The cells were vortexed vigorously and 7 volumes of 4M LiCl added. The mixture was incubated at 4°C
5 for 15-20 hrs. The suspension was spun and the supernatant resuspended in 3M LiCl and centrifuged again. The pellet was dissolved in 2ml 0.1 % SDS, 10 mM Tris HCl and 1 mM EDTA. The suspension was frozen at -20°C, and thawed by vortexing for 20 s every 10 min for 45 min. A large white
10 pellet was left behind and the clear supernatant was extracted with phenol chloroform, then with chloroform. The RNA was precipitated by adding 1/10 volume 3M sodium acetate and 2 vol ethanol and leaving overnight at -20°C. The pellet was suspended in 0.2 ml water and reprecipitated with
15 ethanol. Aliquots for cDNA synthesis were taken from the ethanol precipitate which had been vortexed to create a fine suspension.

100 µl of the suspension was precipitated and dissolved in
20 20 µl water for cDNA synthesis [30] using 10 pmole of a HUFOR primer (see below) in final volume of 50 µl. A sample of 5 µl of the cDNA was amplified as in Example 2 except using the primers for the human VH gene families (see below) using a cycle of 95°C, 60°C and 72°C.

25

The back primers for the amplification of human DNA were designed to match the available human heavy and light chain sequences, in which the different families have slightly different nucleotide sequences at the 5' end. Thus for the
30 human VH genes, the primers Hu2VHIBACK, HuVHIIBACK, Hu2VHIIIBACK and HuVH1VBACK were designed as back primers, and HUJH1FOR, HUJH2FOR and HUJH4FOR as forward primers based entirely in the variable gene. Another set of forward primers Hu1VHFOR, Hu2VHFOR, Hu3VHFOR, and Hu4VHFOR was also
35 used, which were designed to match the human J-regions and the 5' end of the constant regions of different human isotopes.

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One such experiment was analysed in detail to establish whether there was a diverse repertoire in a patient with HIV infection. It is known that during the course of AIDS, that T-cells and also antibodies are greatly diminished in the blood. Presumably the repertoire of lymphocytes is also diminished. In this experiment, for the forward priming, an equimolar mixture of primers Hu1VHFOR, Hu2VHFOR, Hu3VHFOR, and Hu4VHFOR (in PCR 25 pmole of primer 5' ends) was used. For the back priming, the primers Hu2VHIBACK, HuVHIIIBACK, Hu2VHIIIIBACK and HuVHI1VBACK were used separately in four separate primings. The amplified DNA from the separate primings was then pooled, digested with restriction enzymes PstI and BstEII as above, and then cloned into the vector M13VHPCR1 for sequencing. The sequences reveal a diverse repertoire (Fig. 11) at this stage of the disease.

20 For human V κ genes the primers HUJK1FOR, HUJK3FOR, HUJK4FOR and HUJK5FOR were used as forward primers and VK1BACK as back primer. Using these primers it was possible to see a band of amplified ds cDNA of the correct size by gel electrophoresis.

Example 4

Cloning of unrearranged variable gene genomic DNA from human peripheral blood lymphocytes

5

Human peripheral blood lymphocytes of a patient with non-Hodgkins lymphoma were prepared as in Example 3 (Method 1). The genomic DNA was prepared from the PBL using the technique described in Example 2 (Method 2). The VH region
10 in the isolated genomic DNA was then amplified and cloned using the general protocol described in the first two paragraphs of the section headed "Amplification from RNA/DNA hybrid" in Example 1 above, except that during the annealing part of each cycle, the temperature was held at 55°C and
15 that 30 cycles were used. At the end of the 30 cycles, the reaction mixture was held at 60°C for five minutes to ensure that complete elongation and renaturation of the amplified fragments had taken place.

20 The forward primer used was HuHep1FOR, which contains a SacI
site. This primer is designed to anneal to the 3' end of
the unrearranged human VH region gene, and in particular
includes a sequence complementary to the last three codons
in the VH region gene and nine nucleotides downstream of
25 these three codons.

As the back primer, an equimolar mixture of HuOcta1BACK, HuOcta2BACK and HuOcta3BACK was used. These primers anneal to a sequence in the promoter region of the genomic DNA VH gene (see Figure 1). 5µl of the amplified DNA was checked on 2% agarose gels in TBE buffer and stained with ethidium bromide. A double band was seen of about 620 nucleotides which corresponds to the size expected for the unrearranged VH gene. The ds cDNA was digested with SacI and cloned into an M13 vector for sequencing. Although there are some sequences which are identical, a range of different unrearranged human VH genes were identified (Figure 12).

[illegible]

Example 5Cloning Variable Domains with Binding Activities from a Hybridoma

5

The heavy chain variable domain (VHLYS) of the D1.3 (anti-lysozyme) antibody was cloned into a vector similar to that described previously [42] but under the control of the lac z promoter, such that the VHLYS domain is attached to a pelB leader sequence for export into the periplasm. The vector was constructed by synthesis of the pelB leader sequence [43], using overlapping oligonucleotides, and cloning into a pUC 19 vector [35]. The VHLYS domain of the D1.3 antibody was derived from a cDNA clone [44] and the construct (pSW1) 15 sequenced (Figure 13).

To express both heavy and light chain variable domains together, the light chain variable region (VKLYS) of the D1.3 antibody was introduced into the pSW1 vector, with a 20 pelB signal sequence to give the construct pSW2 (Figure 14).

A strain of E. coli (BMH71-18) [45] was then transformed [46,47] with the plasmid pSW1 or pSW2, and colonies resistant to ampicillin (100 µg/ml) were selected on a rich 25 (2 x TY = per litre of water, 16g Bacto-tryptone, 10g yeast extract, 5g NaCl) plate which contained 1% glucose to repress the expression of variable domain(s) by catabolite repression.

30 The colonies were inoculated into 50 ml 2 x TY (with 1% glucose and 100 µg/ml ampicillin) and grown in flasks at 37°C with shaking for 12-16 hr. The cells were centrifuged, the pellet washed twice with 50 mM sodium chloride, resuspended in 2 x TY medium containing 100 µg/ml ampicillin 35 and the inducer IPTG (1 mM) and grown for a further 30 hrs at 37°C. The cells were centrifuged and the supernatant was passed through a Nalgene filter (0.45 µm) and then down a 1 - 5 ml lysozyme-Sepharose affinity column. (The column was

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derived by coupling lysozyme at 10 mg/ml to CNBr activated Sepharose.) The column was first washed with phosphate buffered saline (PBS), then with 50 mM diethylamine to elute the VHLYS domain (from pSW1, or VHLYS in association with VKLYS (from pSW2).

The VHLYS and VKLYS domains were identified by SDS polyacrylamide electrophoresis as the correct size. In addition, N-terminal sequence determination of VHLYS and VKLYS isolated from a polyacrylamide gel showed that the signal peptide had been produced correctly. Thus both the Fv fragment and the VHLYS domains are able to bind to the lysozyme affinity column, suggesting that both retain at least some of the affinity of the original antibody.

The size of the VHLYS domain was compared by FPLC with that of the Fv fragment on Superose 12. This indicates that the VHLYS domain is a monomer. The binding of the VHLYS and Fv fragment to lysozyme was checked by ELISA, and equilibrium and rapid reaction studies were carried out using fluorescence quench.

The ELISA for lysozyme binding was undertaken as follows:

- (1) The plates (Dynatech Immulon) were coated with 200 μ l per well of 300 μ g/ml lysozyme in 50 mM NaHCO_3 , pH 9.6 overnight at room temperature;
- (2) The wells were rinsed with three washes of PBS, and blocked with 300 μ l per well of 1% Sainsbury's instant dried skimmed milk powder in PBS for 2 hours at 37°C;
- (3) The wells were rinsed with three washes of PBS and 200 μ l of VHLYS or Fv fragment (VHLYS associated with VKLYS) were added and incubated for 2 hours at room temperature;
- (4) The wells were washed three times with 0.05% Tween 20 in PBS and then three times with PBS to remove detergent;
- (5) 200 μ l of a suitable dilution (1:1000) of rabbit polyclonal antisera raised against the Fv fragment in 2% skimmed milk powder in PBS was added to each well and incubated at room temperature for 2 hours;

- (6) Washes were repeated as in (4);
- (7) 200 μ l of a suitable dilution (1:1000) of goat anti-rabbit antibody (ICN Immunochemicals) coupled to horse radish peroxidase, in 2% skimmed milk powder in PBS, was added to each well and incubated at room temperature for 1 hour;
- (8) Washes were repeated as in (4); and
- (9) 200 μ l 2,2'-azino-bis(3-ethylbenzthiazolinesulphonic acid) [Sigma] (0.55 mg/ml, with 1 μ l 20% hydrogen peroxide: water per 10 ml) was added to each well and the colour allowed to develop for up to 10 minutes at room temperature.

The reaction was stopped by adding 0.05% sodium azide in 50 mM citric acid pH 4.3. ELISA plates were read in a Titertek Multiscan plate reader. Supernatant from the induced bacterial cultures of both pSW1 (VHLYS domain) or pSW2 (Fv fragment) was found to bind to lysozyme in the ELISA.

The purified VHLYS and Fv fragments were titrated with lysozyme using fluorescence quench (Perkin Elmer LS5B Luminescence Spectrometer) to measure the stoichiometry of binding and the affinity constant for lysozyme [48,49]. The titration of the Fv fragment at a concentration of 30 nM indicates a dissociation constant of 2.8 nM using a Scatchard analysis.

A similar analysis using fluorescence quench and a Scatchard plot was carried out for VHLYS, at a VHLYS concentration of 100 nM. The stoichiometry of antigen binding is about 1 mole of lysozyme per mole of VHLYS (calculated from plot). (The concentration of VH domains was calculated from optical density at 280 nM using the typical extinction coefficient for complete immunoglobulins.) Due to possible errors in measuring low optical densities and the assumption about the extinction coefficient, the stoichiometry was also measured more carefully. VHLYS was titrated with lysozyme as above using fluorescence quench. To determine the concentration of VHLYS a sample of the stock solution was removed, a known

5 12 PM.

[illegible]

Example 6Cloning complete variable domains with binding activities from mRNA or DNA of antibody-secreting cells

5

A mouse was immunised with hen egg white lysozyme (100 µg i.p. day 1 in complete Freund's adjuvant), after 14 days immunised i.p. again with 100 µg lysozyme with incomplete Freund's adjuvant, and on day 35 i.v. with 50 µg lysozyme in saline. On day 39, spleen was harvested. A second mouse was immunised with keyhole limpet haemocyanin (KLH) in a similar way. The DNA was prepared from the spleen according to Example 2 (Method 2). The VH genes were amplified according to the preferred method in Example 2.

15

Human peripheral blood lymphocytes from a patient infected with HIV were prepared as in Example 3 (Method 2) and mRNA prepared. The VH genes were amplified according to the method described in Example 3, using primers designed for human VH gene families.

20

After the PCR, the reaction mixture and oil were extracted twice with ether, once with phenol and once with phenol/CHCl₃. The double stranded DNA was then taken up in 50 µl of water and frozen. 5 µl was digested with PstI and BstEII (encoded within the amplification primers) and loaded on an agarose gel for electrophoresis. The band of amplified DNA at about 350 bp was extracted.

25

30 Expression of anti-lysozyme activities

The repertoire of amplified heavy chain variable domains (from mouse immunised with lysozyme and from human PBLs) was then cloned directly into the expression vector pSW1HPOLYMYC. This vector is derived from pSW1 except that the VHLYS gene has been removed and replaced by a polylinker restriction site. A sequence encoding a peptide tag was inserted (Figure 15). Colonies were toothpicked into 1 ml

35

cultures. After induction (see Example 5 for details), 10 μ l of the supernatant from fourteen 1 ml cultures was loaded on SDS-PAGE gels and the proteins transferred electrophoretically to nitrocellulose. The blot was probed with antibody 9E10 directed against the peptide tag.

The probing was undertaken as follows. The nitrocellulose filter was incubated in 3% bovine serum albumin (BSA)/TBS buffer for 20 min (10 x TBS buffer is 100 mM Tris.HCl, pH 7.4, 9% w/v NaCl). The filter was incubated in a suitable dilution of antibody 9E10 (about 1/500) in 3% BSA/TBS for 1 - 4 hrs. After three washes in TBS (100 ml per wash, each wash for 10 min), the filter was incubated with 1:500 dilution of anti-mouse antibody (peroxidase conjugated anti-mouse Ig (Dakopats)) in 3% BSA/TBS for 1 - 2 hrs. After three washes in TBS and 0.1% Triton X-100 (about 100 ml per wash, each wash for 10 min), a solution containing 10 ml chloronaphthol in methanol (3 mg/ml), 40 ml TBS and 50 μ l hydrogen peroxide solution was added over the blot and allowed to react for up to 10 min. The substrate was washed out with excess water. The blot revealed bands similar in mobility to VHLYSMYC on the Western blot, showing that other VH domains could be expressed.

Colonies were then toothpicked individually into wells of an ELISA plate (200 μ l) for growth and induction. They were assayed for lysozyme binding with the 9E10 antibody (as in Examples 5 and 7). Wells with lysozyme-binding activity were identified. Two positive wells (of 200) were identified from the amplified mouse spleen DNA and one well from the human cDNA. The heavy chain variable domains were purified on a column of lysozyme-Sepharose. The affinity for lysozyme of the clones was estimated by fluorescence quench titration as >50 nM. The affinities of the two clones (VH3 and VH8) derived from the mouse genes were also estimated by stop flow analysis (ratio of k_{on}/k_{off}) as 12 nM and 27 nM respectively. Thus both these clones have a comparable affinity to the VHLYS domain. The encoded amino

acid sequences of of VH3 and VH3 are given in Figure 16, and that of the human variable domain in Figure 17.

A library of VH domains made from the mouse immunised with
5 lysozyme was screened for both lysozyme and keyhole limpet
haemocyanin (KLH) binding activities. Two thousand colonies
were toothpicked in groups of five into wells of ELISA
plates, and the supernatants tested for binding to lysozyme
coated plates and separately to KLH coated plates. Twenty
10 one supernatants were shown to have lysozyme binding
activities and two to have KLH binding activities. A second
expression library, prepared from a mouse immunised with KLH
was screened as above. Fourteen supernatants had KLH
binding activities and a single supernatant had lysozyme
15 binding activity.

This shows that antigen binding activities can be prepared
from single VH domains, and that immunisation facilitates
the isolation of these domains.

Example 7Cloning variable domains with binding activities by mutagenesis.

5

Taking a single rearranged VH gene, it may be possible to derive entirely new antigen binding activities by extensively mutating each of the CDRs. The mutagenesis might be entirely random, or be derived from pre-existing
10 repertoires of CDRs. Thus a repertoire of CDR3s might be prepared as in the preceding examples by using "universal" primers based in the flanking sequences, and likewise repertoires of the other CDRs (singly or in combination). The CDR repertoires could be stitched into place in the
15 flanking framework regions by a variety of recombinant DNA techniques.

CDR3 appears to be the most promising region for mutagenesis as CDR3 is more variable in size and sequence than CDRs 1
20 and 2. This region would be expected to make a major contribution to antigen binding. The heavy chain variable region (VHLYS) of the anti-lysozyme antibody D1.3 is known to make several important contacts in the CDR3 region.

25 Multiple mutations were made in CDR3. The polymerase chain reaction (PCR) and a highly degenerate primer were used to make the mutations and by this means the original sequence of CDR3 was destroyed. (It would also have been possible to construct the mutations in CDR3 by cloning a mixed
30 oligonucleotide duplex into restriction sites flanking the CDR or by other methods of site-directed mutagenesis). Mutants expressing heavy chain variable domains with affinities for lysozyme were screened and those with improved affinities or new specificities were identified.

35

The source of the heavy chain variable domain was an M13 vector containing the VHLYS gene. The body of the sequence encoding the variable region was amplified using the

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polymerase chain reaction (PCR) with the mutagenic primer VHMUT1 based in CDR3 and the M13 primer which is based in the M13 vector backbone. The mutagenic primer hypermutates the central four residues of CDR3 (Arg-Asp-Tyr-Arg). The PCR was carried out for 25 cycles on a Techne PHC-1 programmable heat block using 100 ng single stranded M13mp19SW0 template, with 25 pmol of VHMUT1 and the M13 primer, 0.5 mM each dNTP, 67mM Tris.HCl, pH 8.8, 10 mM MgCl₂, 17 mM (NH₄)₂SO₄, 200 µg/ml gelatine and 2.5 units Taq polymerase in a final volume of 50 µl. The temperature regime was 95°C for 1.5 min, 25°C for 1.5 min and 72°C for 3 min (However a range of PCR conditions could be used). The reaction products were extracted with phenol/chloroform, precipitated with ethanol and resuspended in 10 mM Tris. HCl and 0.1 mM EDTA, pH 8.0.

The products from the PCR were digested with PstI and BstEII and purified on a 1.5% LGT agarose gel in Tris acetate buffer using GeneClean (Bio 101, LaJolla). The gel purified band was ligated into pSW2HPOLY (Figure 19). (This vector is related to pSW2 except that the body of the VHLYS gene has been replaced by a polylinker.) The vector was first digested with BstEII and PstI and treated with calf-intestinal phosphatase. Aliquots of the reaction mix were used to transform E. coli BMH 71-18 to ampicillin resistance. Colonies were selected on ampicillin (100 µg/ml) rich plates containing glucose at 0.8% w/v.

Colonies resulting from transfection were picked in pools of five into two 96 well Corning microtitre plates, containing 200 µl 2 x TY medium and 100 µl TY medium, 100 µg/ml ampicillin and 1% glucose. The colonies were grown for 24 hours at 37°C and then cells were washed twice in 200 µl 50 mM NaCl, pelleting the cells in an IEC Centra-3 bench top centrifuge with microtitre plate head fitting. Plates were spun at 2,500 rpm for 10 min at room temperature. Cells were resuspended in 200 µl 2 x TY, 100 µg/ml ampicillin and 1 mM IPTG (Sigma) to induce expression, and grown for a

further 24 hr.

Cells were spun down and the supernatants used in ELISA with lysozyme coated plates and anti-idiotypic sera (raised in 5 rabbits against the Fv fragment of the D1.3 antibody). Bound anti-idiotypic serum was detected using horse radish peroxidase conjugated to anti-rabbit sera (ICN Immunochemicals). Seven of the wells gave a positive result in the ELISA. These pools were restreaked for single 10 colonies which were picked, grown up, induced in microtitre plates and rescreened in the ELISA as above. Positive clones were grown up at the 50 ml scale and expression was induced. Culture supernatants were purified as in Example 5 on columns of lysozyme-Sepharose and eluates analysed on 15 SDS-PAGE and staining with Page Blue 90 (BDH). On elution of the column with diethylamine, bands corresponding to the VHLYS mutant domains were identified, but none to the VKLYS domains. This suggested that although the mutant domains could bind to lysozyme, they could no longer associate with 20 the VKYLS domains.

For seven clones giving a positive reaction in ELISA, plasmids were prepared and the VKLYS gene excised by cutting with EcoRI and religating. Thus the plasmids should only 25 direct the expression of the VHLYS mutants. 1.5 ml cultures were grown and induced for expression as above. The cells were spun down and supernatant shown to bind lysozyme as above. (Alternatively the amplified mutant VKLYS genes could have been cloned directly into the pSWIHPOLY vector 30 for expression of the mutant activities in the absence of VKLYS.)

An ELISA method was devised in which the activities of bacterial supernatants for binding of lysozyme (or KLH) were 35 compared. Firstly a vector was devised for tagging of the VH domains at its C-terminal region with a peptide from the c-myc protein which is recognised by a monoclonal antibody 9E10. The vector was derived from pSW1 by a BstEII and SmaI

double digest, and ligation of an oligonucleotide duplex made from

5' GTC ACC GTC TCC TCA GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT TAA TAA 3' and

5' TTA TTA ATT CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC TGA GGA GAC G 3'.

The VHLYSMYC protein domain expressed after induction was shown to bind to lysozyme and to the 9E10 antibody by ELISA as follows:

10 (1) Falcon (3912) flat bottomed wells were coated with 180 μ l lysozyme (3 mg/ml) or KLH (50 μ g/ml) per well in 50 mM NaHCO₃, pH 9.6, and left to stand at room temperature overnight;

(2) The wells were washed with PBS and blocked for 2 hrs at 37°C with 200 μ l 2% Sainsbury's instant dried skimmed milk powder in PBS per well;

(3) The Blocking solution was discarded, and the walls washed out with PBS (3 washes) and 150 μ l test solution (supernatant or purified tagged domain) pipetted into each well. The sample was incubated at 37°C for 2 hrs;

(4) The test solution was discarded, and the wells washed out with PBS (3 washes). 100 μ l of 4 μ g/ml purified 9E10 antibody in 2% Sainsbury's instant dried skimmed milk powder in PBS was added, and incubated at 37°C for 2 hrs;

25 (5) The 9E10 antibody was discarded, the wells washed with PBS (3 washes). 100 μ l of 1/500 dilution of anti-mouse antibody (peroxidase conjugated anti-mouse Ig (Dakopats)) was added and incubated at 37°C for 2 hrs;

(6) The second antibody was discarded and wells washed three times with PBS; and

30 (7) 100 μ l 2,2'-azino-bis(3-ethylbenzthiazolinesulphonic acid) [Sigma] (0.55 mg/ml, with 1 μ l 20% hydrogen peroxide: water per 10 ml) was added to each well and the colour allowed to develop for up to 10 minutes at room temperature.

35

The reaction was stopped by adding 0.05% sodium azide in 50 mM citric acid, pH 4.3. ELISA plates were read in an Titertek Multiscan plate reader.

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The activities of the mutant supernatants were compared with VHLYS supernatant by competition with the VHLYSMYC domain for binding to lysozyme. The results show that supernatant from clone VHLYSMUT59 is more effective than wild type VHLYS supernatant in competing for VHLYSMYC. Furthermore, Western blots of SDS-PAGE aliquots of supernatant from the VHLYS and VHLYSMUT59 domain (using anti-Fv antisera) indicated comparable amounts of the two samples. Thus assuming identical amounts of VHLYS and VHLYSMUT59, the affinity of the mutant appears to be greater than that of the VHLYS domain.

To check the affinity of the VHLYSMUT59 domain directly, the clone was grown at the 11 scale and 200-300 μ g purified on lysozyme-Sepharose as in Example 5. By fluorescence quench titration of samples of VHLYS and VHLYSMUT59, the number of binding sites for lysozyme were determined. The samples of VHLYS and VHLYSMUT59 were then compared in the competition ELISA with VHLYSMYC over two orders of magnitude. In the competition assay each microtitre well contained a constant amount of VHLYSMYC (approximately 0.6 μ g VHLYSMYC). Varying amounts of VHLYS or VHLYSMUT59 (3.8 μ M in lysozyme binding sites) were added (0.166 - 25 μ l). The final volume and buffer concentration in all wells was constant. 9E10 (anti-myc) antibody was used to quantitate bound VHLYSMYC in each assay well. The % inhibition of VHLYSMYC binding was calculated for each addition of VHLYS or VHLYSMUT59, after subtraction of background binding. Assays were carried out in duplicate. The results indicate that VHLYSMUT59 has a higher affinity for lysozyme than VHLYS.

The VHLYSMUT59 gene was sequenced (after recloning into M13) and shown to be identical to the VHLYS gene except for the central residues of CDR3 (Arg-Asp-Tyr-Arg). These were replaced by Thr-Gln-Arg-Pro: (encoded by ACACAAAGGCCA).

A library of 2000 mutant VH clones was screened for lysozyme and also for KLH binding (toothpicking 5 colonies per well as described in Example 6). Nineteen supernatants were identified with lysozyme binding activities and four with
5 KLH binding activities. This indicates that new specificities and improved affinities can be derived by making a random repertoire of CDR3.

Example 8

10

Construction and expression of double domain for lysozyme binding.

The finding that single domains have excellent binding
15 activities should allow the construction of strings of domains (concatamers). Thus, multiple specificities could be built into the same molecule, allowing binding to different epitopes spaced apart by the distance between domain heads. Flexible linker regions could be built to
20 space out the domains. In principle such molecules could be devised to have exceptional specificity and affinity.

Two copies of the cloned heavy chain variable gene of the D1.3 antibody were linked by a nucleotide sequence encoding
25 a flexible linker

Gly-Gly-Gly-Ala-Pro-Ala-Ala-Pro-Ala-Gly-Gly-Gly-
(by several steps of cutting, pasting and site directed mutagenesis) to yield the plasmid pSW3 (Figure 20). The expression was driven by a lacZ promoter and the protein was
30 secreted into the periplasm via a pelB leader sequence (as described in Example 5 for expression of pSW1 and pSW2). The protein could be purified to homogeneity on a lysozyme affinity column. On SDS polyacrylamide gels, it gave a band of the right size (molecular weight about 26,000). The
35 protein also bound strongly to lysozyme as detected by ELISA (see Example 5) using anti-idiotypic antiserum directed against the Fv fragment of the D1.3 antibody to detect the protein. Thus, such constructs are readily made and secreted

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Example 9

By purification on an affinity column of lysozyme Sepharose
15 it was shown that the VHLYS-Cys domain was expressed in
association with the VKLYS variable domain, but the overall
yields were much lower than the wild type Fv fragment.
Comparison of non-reducing and reducing SDS polyacrylamide
gels of the purified Fv-Cys protein indicated that the two
20 VH-Cys domains had become linked through the introduced
cysteine residue.

Linking of enzyme activities to VH domains should be possible by either cloning the enzyme on either the N-terminal or the C-terminal side of the VH domain. Since both partners must be active, it may be necessary to design a suitable linker (see Example 8) between the two domains. For secretion of the VH-enzyme fusion, it would be preferable to utilise an enzyme which is usually secreted. In Figure 21, there is shown the sequence of a fusion of a VH domain with alkaline phosphatase. The alkaline phosphatase gene was cloned from a plasmid carrying the *E. coli* alkaline phosphatase gene in a plasmid pEK48 [51] using the polymerase chain reaction. The gene was amplified with

the primers

5' CAC CAC GGT CAC CGT CTC CTC ACG GAC ACC AGA AAT GCC TGT
TCT G 3' and

5' GCG AAA ATT CAC TCC CGG GCG CGG TTT TAT TTC 3'. The gene
5 was introduced into the vector pSW1 by cutting at BstEII and
SmaI. The construction (Figure 21) was expressed in *E. coli*
strain BMH71-18 as in Example 5 and screened for phosphatase
activity using 1 mg/ml p-nitrophenylphosphate as substrate
in 10mM diethanolamine and 0.5 mM MgCl₂, pH 9.5) and also on
10 SDS polyacrylamide gels which had been Western blotted
(detecting with anti-idiotypic antiserum). No evidence was
found for the secretion of the linked VHLYS-alkaline
phosphatase as detected by Western blots (see Example 5), or
for secretion of phosphatase activity.

15

However when the construct was transfected into a bacterial
strain BL21DE3 [52] which is deficient in proteases, a band
of the correct size (as well as degraded products) was
detected on the Western blots. Furthermore phosphatase
20 activity could now be detected in the bacterial supernatant.
Such activity is not present in supernatant from the strain
which had not been transfected with the construct.

A variety of linker sequences could then be introduced at
25 the BstEII site to improve the spacing between the two
domains.

Example 11

30 Coexpression of VH domains with Vk repertoire

A repertoire of V_k genes was derived by PCR using primers as
described in Example 2 from DNA prepared from mouse spleen
and also from mouse spleen mRNA using the primers VK3FOR and
35 VK2BACK and a cycle of 94°C for 1 min, 60°C for 1 min, 72°C
for 2 min. The PCR amplified DNA was fractionated on the
agarose gel, the band excised and cloned into a vector which
carries the VHLYS domain (from the D1.3 antibody), and a

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cloning site (SacI and XhoI) for cloning of the light chain variable domains with a myc tail (pSW1VHLYS-VKPOLYMYC, Figure 22).

5 Clones were screened for lysozyme binding activities as described in Examples 5 and 7 via the myc tag on the light chain variable domain, as this should permit the following kinds of V κ domains to be identified:

- 10 (1) those which bind to lysozyme in the absence of the VHLYS domain;
- (2) those which associate with the heavy chain and make no contribution to binding of lysozyme; and
- (3) those which associate with the heavy chain and also contribute to binding of lysozyme (either helping or 15 hindering).

This would not identify those V κ domains which associated with the VHLYS domain and completely abolished its binding to lysozyme.

20

In a further experiment, the VHLYS domain was replaced by the heavy chain variable domain VH3 which had been isolated from the repertoire (see Example 6), and then the V κ domains cloned into the vector. (Note that the VH3 domain has an 25 internal SacI site and this was first removed to allow the cloning of the V κ repertoire as SacI-XhoI fragments.)

By screening the supernatant using the ELISA described in Example 6, bacterial supernatants will be identified which 30 bind lysozyme.

Example 12High expression of VH domains.

5 By screening several clones from a VH library derived from
a mouse immunised with lysozyme via a Western blot, using
the 9E10 antibody directed against the peptide tag, one
clone was noted with very high levels of expression of the
domain (estimated as 25 - 50 mg/l). The clone was sequenced
10 to determine the nature of the sequence. The sequence proved
to be closely related to that of the VHLYS domain, except
with a few amino acid changes (Figure 23). The result was
unexpected, and shows that a limited number of amino acid
changes, perhaps even a single amino acid substitution, can
15 cause greatly elevated levels of expression.

By making mutations of the high expressing domain at these
residues, it was found that a single amino acid change in
the VHLYS domain (Asn 35 to His) is sufficient to cause the
20 domain to be expressed at high levels.

CONCLUSION

It can thus be seen that the present invention enables the
25 cloning, amplification and expression of heavy and light
chain variable domain encoding sequences in a much more
simple manner than was previously possible. It also shows
that isolated variable domains or such domains linked to
effector molecules are unexpectedly useful.

30

It will be appreciated that the present invention has been
described above by way of example only and that variations
and modifications may be made by the skilled person without
departing from the scope of the invention.

00334-43300

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CLAIMS

1. A single domain ligand consisting of at least part of the variable domain of one chain of a molecule from the immunoglobulin (Ig) superfamily.
2. The ligand of claim 1, which consists of the variable domain of an Ig heavy chain.
3. The ligand of claim 1, which consists of the variable domain of an Ig chain with one or more point mutations from the natural sequence.
4. A receptor comprising a ligand of any one of claims 1 to 3 linked to one or more of an effector molecule, a prosthetic group, a label, a solid support or one or more other ligands having the same or different specificity.
5. The receptor of claim 4, comprising at least two ligands.
6. The receptor of claim 5, wherein the first ligand binds to a first epitope of an antigen and the second ligand binds to a second epitope.
7. The receptor of claim 6, which includes an effector molecule or label.
8. The receptor of any one of claims 5 to 7 which comprises a ligand and another protein molecule, produced by recombinant DNA technology as a fusion product.
9. The receptor of claim 8, wherein a linker peptide sequence is placed between the ligand and the other protein molecule.
10. A method of cloning a sequence (the target sequence) which encodes at least part of the variable domain of an Ig

superfamily molecule, which method comprises:

- (a) providing a sample of double stranded (ds) nucleic acid which contains the target sequence;
 - (b) denaturing the sample so as to separate the two
5 strands;
 - (c) annealing to the sample a forward and a back oligonucleotide primer, the forward primer being specific for a sequence at or adjacent the 3' end of the sense strand of the target sequence, the back primer being specific for
10 a sequence at or adjacent the 3' end of the antisense strand of the target sequence, under conditions which allow the primers to hybridise to the nucleic acid at or adjacent the target sequence;
 - (d) treating the annealed sample with a DNA polymerase
15 enzyme in the presence of deoxynucleoside triphosphates under conditions which cause primer extension to take place; and
 - (e) denaturing the sample under conditions such that the extended primers become separated from the target sequence.
20
11. The method of claim 10, further including the step (f) of repeating steps (c) to (e) on the denatured mixture a plurality of times.
 - 25 12. The method of claim 10 or claim 11, which is used to clone a complete variable domain from an Ig heavy chain.
 13. The method of claim 10 or claim 11 which is used to produce a DNA sequence encoding a ligand according to any
30 one of claims 1 to 3.
 14. The method of any one of claims 10 to 13, wherein the forward and back primers are provided as single oligonucleotides.
35
 15. The method of any one of claims 10 to 13, wherein the forward and back primers are each supplied as a mixture of closely related oligonucleotides.

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16. The method of claim 14 or claim 15, wherein the primers which are used are species specific general primers.

5 17. The method of any one of claims 10 to 16, wherein the ds nucleic acid sequence is genomic DNA.

18. The method of any one of claims 10 to 17, wherein the ds nucleic acid is derived from a human.

10

19. The method of any one of claims 10 to 18, wherein the ds nucleic acid is derived from peripheral blood lymphocytes.

15 20. The method of any one of claims 10 to 18, wherein each primer includes a sequence encoding a restriction enzyme recognition site.

21. The method of claim 20, wherein the restriction enzyme
20 recognition site is located in the sequence which is annealed to the ds nucleic acid.

22. The method of any one of claims 10 to 21, wherein the product ds cDNA is inserted into an expression vector and
25 expressed alone.

23. The method of any one of claims 10 to 22, wherein the product ds cDNA is expressed in combination with a complementary variable domain.

30

24. The method of any one of claims 10 to 23, wherein the cloned ds cDNA is inserted into an expression vector already containing sequences encoding one or more constant domains to allow the vector to express Ig-type chains.

35

25. The method of any one of claims 10 to 24, wherein the cloned ds cDNA is inserted into an expression vector so that it can be expressed as a fusion protein.

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26. The method of claim 10, wherein one or both of the primers comprises a mixture of oligonucleotides of hypervariable sequence, whereby a mixture of variable domain
5 encoding sequences is produced.

27. A method of cloning a sequence (the target sequence) which encodes at least part of the variable domain of an Ig superfamily molecule, which method comprises:

10 (a) providing a sample of double stranded (ds) nucleic acid which contains the target sequence;
(b) denaturing the sample so as to separate the two strands;

(c) annealing to the sample a forward and a back
15 oligonucleotide primer, the forward primer being specific for a sequence at or adjacent the 3' end of the sense strand of the target sequence, the back primer being specific for a sequence at or adjacent the 3' end of the antisense strand of the target sequence, under conditions which allow the
20 primers to hybridise to the nucleic acid at or adjacent the target sequence;

(d) treating the annealed sample with a DNA polymerase enzyme in the presence of deoxynucleoside triphosphates under conditions which cause primer extension to take place;
25 (g) treating the sample of ds cDNA with traces of DNase in the presence of DNA polymerase I to allow nick translation of the DNA; and
(h) cloning the ds cDNA into a vector.

30 28. The method of claim 27, which further includes the steps of:

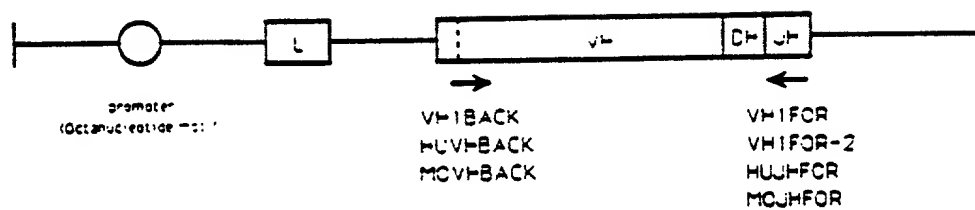
(i) digesting the DNA of recombinant plasmids to release DNA fragments containing genes encoding variable domains; and
35 (j) treating the fragments in a further set of steps (c) to (h).

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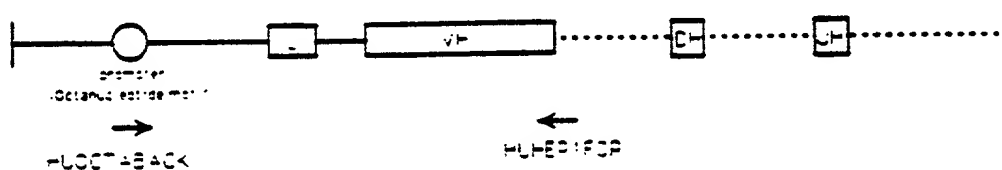
5 30. The method of any one of claims 27 to 29, wherein the product ds cDNA is cloned directly into an expression vector.

32. A primer or mixture of primers according to claim 27,
15 wherein each primer includes a restriction enzyme
recognition site within the sequence which anneals to the
coding part of the variable domain encoding sequence.

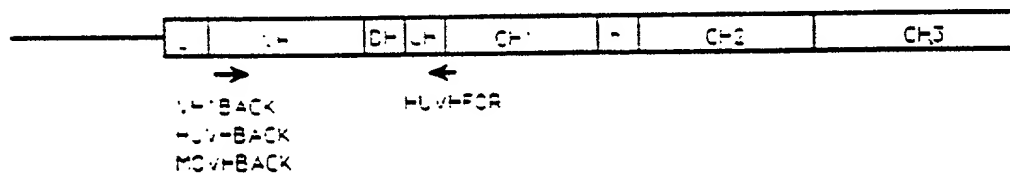
Rearranged heavy chain variable gene (DNA)



Unrearranged heavy chain variable gene (DNA)



Rearranged heavy chain variable gene (mRNA)



Rearranged light chain variable gene (DNA)

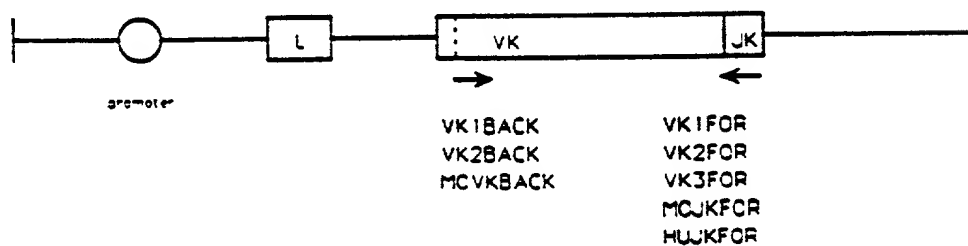


FIG. 1

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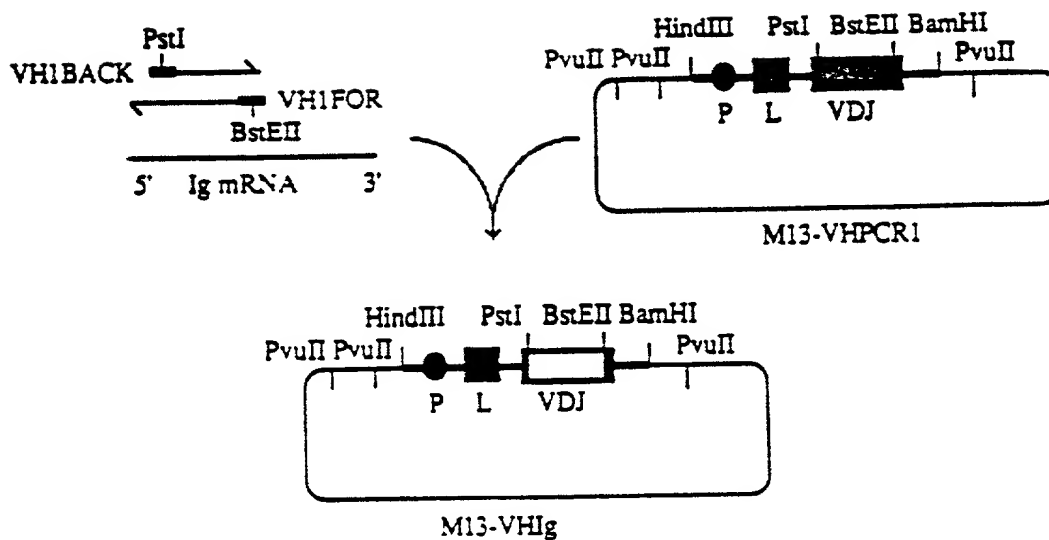


FIG. 2

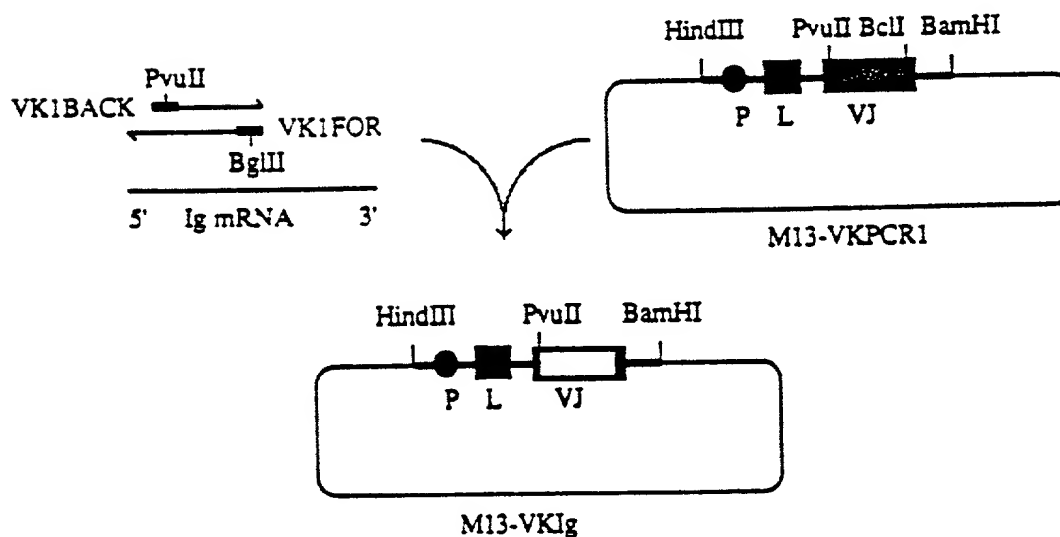


FIG. 4

HLS 22117

AAGGATATGAATATGCAATGCTGTGAATCTACATGGTAAATATAGGTTGTCTATACCA
10 20 30 40 50 60

CAAACAGAAAACATGAGTACAGTTCTCTACAGTTACTGAGCACACAGGACCTCAC
70 80 90 100 110 120

M G W S C : I L F L V A T A T
CATGGGATGGAGCTGTATCAAGCTCTTTTGGTAGCAACAGCTACAGGTAAGGGGCTCAC
130 140 150 160 170 180

AGTAGCAGGCTTGAGGTTTGACATAATATGGGTGACATGACATCCACTTTCCTTC

PstI
 51 10
 G V H S Q V Q L Q E S G P G L V R P
 TTTCCACAGGTTCTCCATCGAGGTCTCACTGAGAGAGCGGTCCAGGTTCTGTGAGAC
 250 255 270 280 290 300

CDR1

15 20 25 30
S Q T L S T C T V S G S T F S S Y W M
TTAGCCAGACCGCTGACCGCTGACCGCTGTCGGCAGCACCTTCAGCAGCTACTGGA
110 120 130 140 150 160

CDR2

35 40 45 50
H W V R Q P P G R G L E W I G R I D P N
TGCAC TGGGT GAGAC AGCCAC CCGAC CAGCT CTTG AGTGG ATTGGA AGGATT GATCT CTA
370 380 390 400 410 420

55 60 65 70
 S G G T K Y N E K F K S R V T M L V D A T
 ATAGTGGTGGTACTAAGTCAATGAGAGTTCAGAGCAGAGTGACAATGCTGGTAGACA
 430 440 450 460 470 480
 75 80 85 90
 S K A N Q F S L R L S S V T A A D T A V Y
 CCAGCAAGAACCAAGTTCAGCGGTGAGACTCAGCAGCGGTGACAGCGCGGACACCGGGTCT
 490 500 510 520 530 540

CDR3

95 100 105 110

Y C A R Y D Y Y G S S Y F D Y W G Q G T

ATTATTGTGCAAGATACGATTACTACGGTAGTAGCTACTTTGACTCTGGGGCCAAGGGA

550 560 570 580 590 600

BstEII
115 1 120
T V T V S S
CCACGGTACCGCTCTCTCTCAGGTGAGTCCCTTACAACCTCTCTCTCTATTTCAGCTTAAT
610 620 630 640 650 660

AGA TTTT ACT GCA TTTG TGGGGGGG AATGTGTGATCTGAA TTT CAGGT CATGAAGGA
670 680 690 700 710 720

CTAGGGACACCTTGGGAGTCAGAAAGGGTCATTGGGAGCCCGGGCTGATGCAGACAGACA

TCTCTGCTCCGAGACTTCTATGCGAGGATTTATAG
 790 800 810

FIG. 3

ML3 VxPCR1

Hand 255

AAGGTAGAAATGGAACTTCACTACCGTAAAGGTTAACA
38 48 58 68 78 88

CAAACAGAAAACATGAGATCACAGTTTCTTTAAGTTACTGAGCAACAGGACCTTAC
98 105 112 129 138 148

M G W S C T T L F L V A T A T
CATGGGATGGAGCTGATCAAGGCTCTCTGGTACACAGCTACAGGTAAAGGGGCTAC
158 168 178 188 198 208

AGTAGCAGGCTTGAGGCTGGACATATAAGGTGACATGACATCCACTTTCGCTTTC
218 228 238 248 258 268

25. 22

G V H S D : Q L : Q S P S S L S A S
 TTTCCACAGTCTCCACTTGGGACA TGAATGATCTTCAGAGCCCAAGCAGCGCTGAGCGCCA
 278 288 298 308 318 328

15 20 25 30
 V G D R V T T C R A S G N I H N Y L A
 GCGTGGGTGACAGAGTACCGTCACTCTGTGAGAGCCAGCGGTACATCCACAACCTACCTGG
 338 345 352 368 378 388

CDR2

35 40 45 50
W Y Q Q K P G K A P K L L T Y Y T T T L
CTTGGTACCACAGCAAGCCAGGTAAGGCTCCAAAGTCTGATCTACTACCAACCAACCC
398 408 418 428 438 448

55 60 65 70
 A D G G V P S R F S G S G S G T D F T F T
 TGGCTGACGGGTGCCAAAGCAGATTACGGGTACGGGTACCGGTTACGCTTCA
 458 468 478 488 498 508

CDR3

75 80 85 90

I S S L Q P E D I A T Y Y C Q H F W S T

CCATCAGCAGCGCTCCAGCCAGGACATGGCCACTACTGCCAGCACTTGTGGAGCA

518 528 538 548 558 568

Bcl I (requires dam⁺ host)

95 100 105 108
 P R T F G Q G T K V V I K R
 CCCCAGGACGCTTCGGGACAGGGACCAAGGTGGGATCAACGTCAGTAGAAATTAAC
 578 588 598 608 618 628

Безум

678 648

FIG. 5

Splice -1
↓ G V H S
AGGTGTCCACTCC

Sequence of ME-1 VK

```

                                Splice                               -1
                                ↓ G V H S
                                AGGTGTCCTACTCC
                                20
1      PvuII                      10
D : Q : T Q S P P S L T V S V G E R V T
GACATTCAGCTTACCCAGCTGCTCCACCATCCCTGACTGTGTCTAGGAGAGAGGGGTCACT
VKI BACK SITE

                                2'A B C D E F          CDR1:
I S C K S N Q N L L W S G N R R Y C L G
ATCAGTTGCAAAATCCCAATCAGAATCTTTTATGGAGTGGAAACCGAAGGTACTGTTTGGGC
35                      40                      50          CDR2
W H Q W K P G Q T P T F L I T W T S D R
TGGCACCAGTGGAAACCCAGGGCAAACTCCTACACCGTTGTATCACTGGGACATCTGTATAGG
                                60                      70
F S G V P D R F I G S G S V T D F T L T
TTCTCTGGAGTCCCTTGATCTTTTCATAGGCAGTGGATCTGTGACAGATTTCATCTGACC
                                80                      90          CDR3
I S S V Q A E D V A V Y F C Q Q H L D L
ATCAGCAGTGTGCGAGGCTGAAGATGTGGCAGTTTATTTCTGTGACGACCATTTGGACCTT
95                      100          BglII/BclI Splice
P Y T F G G G T K L E I K ↓
CCGTACACGTTCCGAGGGGGGACCAAGCTGGAGATCAAACTGAG
                                VKI FOR SITE

```

FIG. 6.

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α -Lys 30

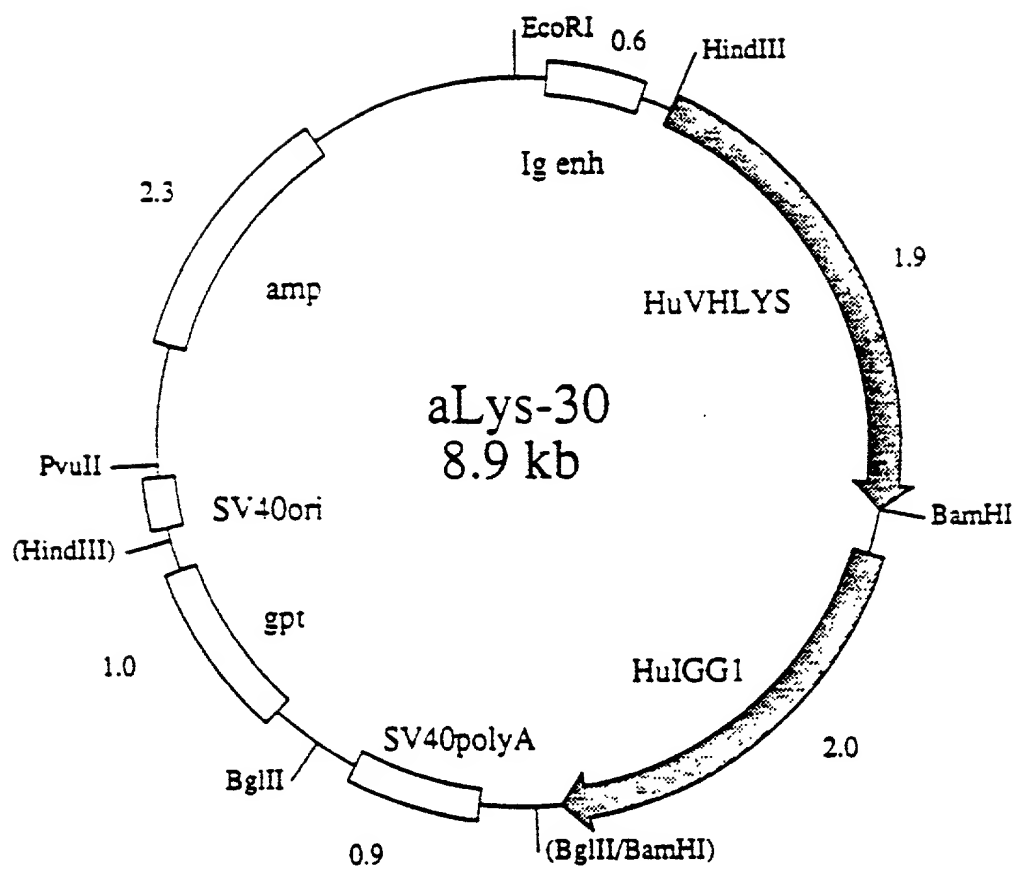


FIG. 7

α -Lys 17

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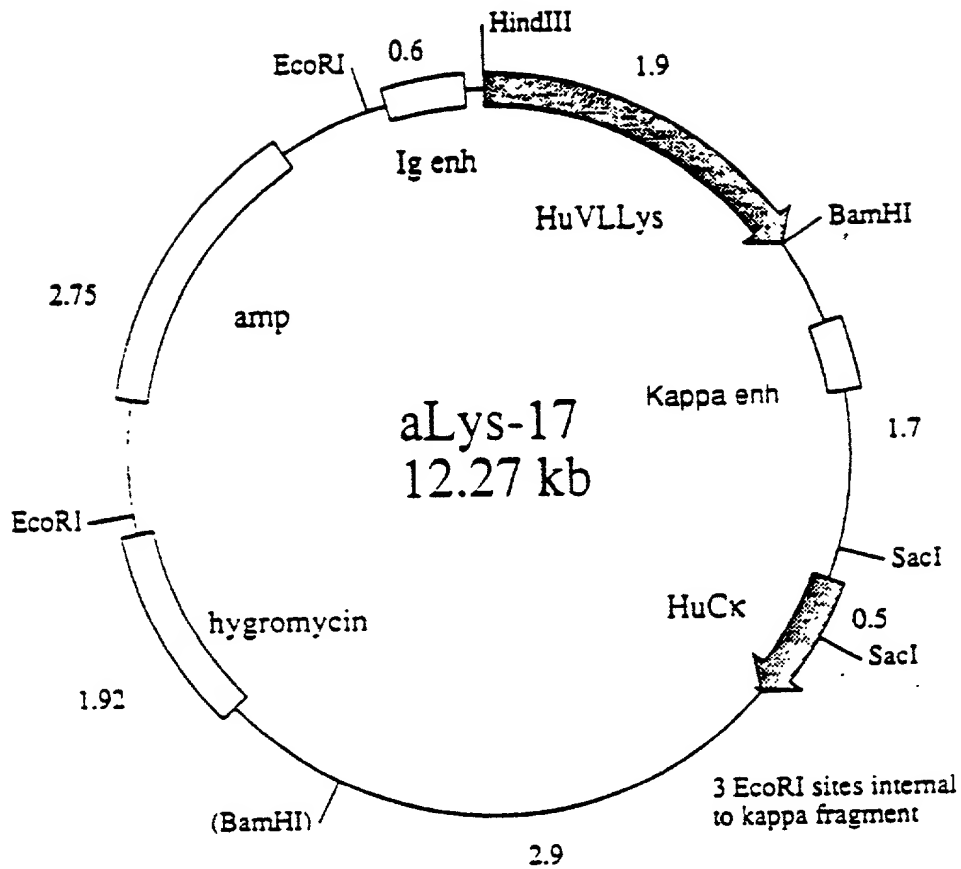


FIG. 8

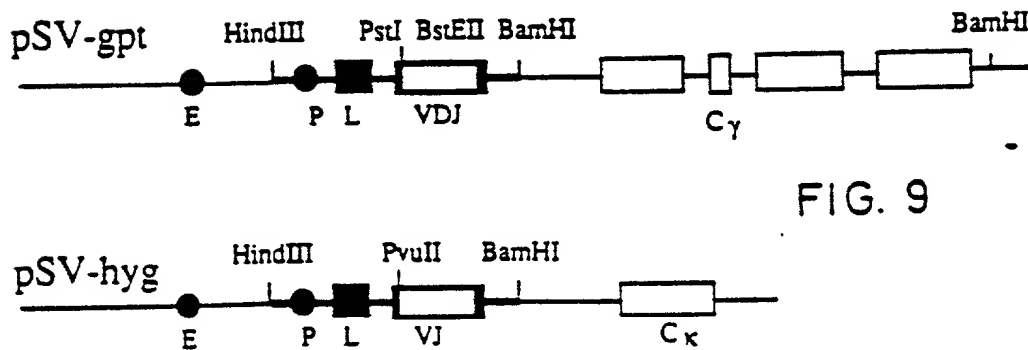


FIG. 9

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ER1

CDR 1

ER2

CDR 2

KABAT 1A

AGT	PGLVKPSQSLSLTGSIYGVST	SGYYN	WVRQFPKGLKLEW	YISYGGSNYNPSLKN
AGS	PGLVKPSQSLSLTGSIYGVST	SGYYN	WVRQSPKGLKLEW	YITHSGSTFYNPSLGS
EGS	PGLVKPSQSLSLTGSIYGVST	SGYYN	WVRQFPKGLKLEW	YISYGGSNYNPSLKN
GGT	PGLVKPSQSLSLTGSIYGVST	SGYYN	WVRQFPKGLKLEW	YISYGGSNYNPSLKN

KABAT 1B

AGG	PVLVAPPSQSLSITGTVSGFSLT	NYGV	WVRQFPKGLKLEW	VWAGGSTNYNSALMS
25GGT	PGLVKPSQSLSITGTVSGFSLT	SYGV	WVRQSPKGLKLEW	VWAGGSTNYNAAFIS
EGS	PGLVKPSQSLSITGTVSGFSLT	SYGV	WVRQFPKGLKLEW	VWAGGSTNYNSALMS
GGG	PGLVKPSQSLSITGTVSGFSLT	SYGV	WVRQSPKGLKLEW	VWAGGSTNYNAAFIS
HGS	PVLVAPPSQSLSITGTVSGFSLT	SYGV	WVRQFPKGLKLEW	VWAGGSTNYNSALMS
25GGS	PGLVKPSQSLSITGTVSGFSLT	SYAG	WVRQFPKGLKLEW	VWAGGSTNYNSALKS
ALZ	PGLVKPSQSLSITGTVSGFSLT	SYAG	WVRQFPKGLKLEW	VWAGGSTNYNSALKS
AGE	PGLVKPSQSLSITGTVSGFSLT	SYGV	WVRQFPKGLKLEW	*****GSTNYNSALKS
25GGG	PGLVKPSQSLSITGTVSGFSLT	SYGV	WVRQSPKGLKLEW	VWAGGSTNYNSALKS
AGS	PGLVKPSQSLSITGTVSGFSLT	SYGV	WVRQSPKGLKLEW	VWAGGSTNYNAAFIS
GGT	PVLVAPPSQSLSITGTVSGFSLT	SYGV	WVRQFPKGLKLEW	VWAGGSTNYNSALMS
HGS	PGLVKPSQSLSITGTVSGFSLT	SYGV	WVRQSPKGLKLEW	VWAGGSTNYNSALKS

KABAT 1IA

EGG	PELLRFPDYSIKISGFGSGYTF	DYAM	WVQSHAPGLEW	VISTYYGDSYNNQKFK
AGT	PELLRFPDYSIKISGFGSGYTF	DYAM	WVQSHAPGLEW	VISTYYGDSYNNQKFK

KABAT 1IB

AGG	AEILVPGASVYLSCKASGYTF	SYAM	WVQFPQGGLEW	EIDPDSSTNYNNQKFK
EGG	AEILVPGASVYLSCKASGYTF	SYAM	WVQFPQGGLEW	EIDPDSSTNYNEKFKS
GGG	AEILVPGASVYLSCKASGYTF	SYAM	WVQFPQGGLEW	RIDPNSGGTKYNEKFKS
GGG	AEILVPGASVYLSCKASGYTF	SYAM	WVQFPQGGLEW	EIDPNSGGTKYNEKFKS
GGG	AEILVPGASVYLSCKASGYTF	SYAM	WVQFPQGGLEW	EIDPNSGGTKYNEKFKS
GGG	AEILVPGASVYLSCKASGYTF	SYAM	WVQFPQGGLEW	EIDPNSGGTKYNEKFKS
EGG	AEILVPGASVYLSCKASGYTF	DYAM	WVQFPQGGLEW	AIDPETGGTAYNNQKFK
GGG	AEILVPGASVYLSCKASGYTF	DYAM	WVQFPQGGLEW	WIDPDSGNTKYNEKFKS
GGG	AEILVPGASVYLSCKASGYTF	SYAM	WVQFPQGGLEW	RIDPDSSTNYNNQKFK
25GGG	AEILVPGASVYLSCKASGYTF	TYPI	WVQFNHGLEW	NIDPYNDDTKYNEKFKS
GGG	AEILVPGASVYLSCKASGYTF	SYAM	WVQFPQGGLEW	NIDPNSGGTKYNNQKFK
HGS	AEILVPGASVYLSCKASGYTF	SYAM	WVQFPQGGLEW	NIDPDSSTNYNNQKFK
GGG	AEILVPGASVYLSCKASGYTF	SYAM	WVQFPQGGLEW	EIDPDSSTNYNNQKFK
25GGG	PELLRFPDYSIKISGFGSGYTF	NYAM	WVQFPQGGLEW	QIDPASGSITYNEMHKO
GGG	AEILVPGASVYLSCKASGYTF	SYAM	WVQFPQGGLEW	EIDPDSSTNYNEKFKS
GGG	AEILVPGASVYLSCKASGYTF	RYAM	WVQFPQGGLEW	SPTMYSDATEYSENFKG
GGG	AEILVPGASVYLSCKASGYTF	SYAM	WVQFPQGGLEW	EIDPDSSTNYNEKFKS

KABAT 1II A

25GGG	GGLVKPSQSLSLSCAASGFTF	DYAM	WVRQFPKALEW	FIRNKANGYTTEYSASVKG
GGG	GGLVKPSQSLSLSCAASGFTF	DYAM	WVRQFPKALEW	LIRNKANGYTTEYSASVKG
GGG	GGLVKPSQSLSLSCAASGFTF	DYAM	WVRQFPKALEW	LIRNKANGYTTEYSASVKG

KABAT 1II B

GGG	GGLVKPSQSLSLSCAASGFTF	DYAM	WVRQFPKALEW	YISSGSSTYYADTVKG
GGG	GGLVKPSQSLSLSCAASGFTF	SHAM	WV*****VA	AISGSGSTYYADTVKG
GGG	GGLVKPSQSLSLSCAASGFTF	SYAM	WVA*APKGLKLEW	AISGSGSTYYADSVKG
GGG	GGLVKPSQSLSLSCAASGFTF	SYAM	WVA*APKGLKLEW	AISGSGSTYYADSVKG
GGG	GGLVKPSQSLSLSCAASGFTF	*****	WVRQFPKALEW	AISGSGSTYYADTVKG
GGG	GGLVKPSQSLSLSCAASGFTF	SYAM	WVA*APKGLKLEW	AISGSGSTYYADSVKG
GGG	GGLVKPSQSLSLSCAASGFTF	*HDM	WVRQSGE*LEW	AISGSGSTYYADTVKG
GGG	GGLVKPSQSLSLSCAASGFTF	SYAM	WVRQFPKALEW	YISSGSSTYYADSVKG

KABAT 1II C

GGG	GGLVKPSQSLSLSCAASGFTF	SHAM	WVRQFPKALEW	EIRNKANGYTTEYSASVKG
-----	-----------------------	------	-------------	---------------------

KABAT 1VA

GGG	AEILVPGASVYLSCKASGYTF	SYAM	WVQFPQGGLEW	EIDPDSSTNYNEKFKS
-----	-----------------------	------	-------------	------------------

FIG. 10a

SUBSTITUTE SHEET

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ZSNDZUFAY
 ZRCH:GFWFAY
 ZSSGSZFY
 ZSSGSZFY

HEDSSGYFY
 HGSY
 LGROYANDY
 KRDYXDRGYYYANDY
 YYDSFFAY
 EGYFFAY
 :YYGSSDYANDY
 11 00.
 21 00.
 28 00.
 37 00.
 42 00.

Ps.gene/Unproductive
Unproductive
Unproductive
Unproductive
Unproductive

40	33	Unproductive
45	33	Unproductive

PSLYTAYC	
MYNMYTGY	
PNWNYNYNYGCL	
LYVYVAYC	
SSGYCY	
GAARATNAY	
GGFYAY	
SPGYC	
SVPGGGFYAYCY	
KQVYGGSLNATAY	
LYVAYCY	
KQGYSTYCYCH	
TGTYFYAY	
24 nt.	Ps.gene
9 nt.	Ps.gene/Unproductive
23 nt.	Unproductive
15 nt.	Unproductive

MILEAGE
 244405SY:AMC
 23 25

AKFRLYDF	Ps.gene
REGVWESLQGV	Ps.gene
RGLHWDF	Ps.gene
RNYGSSPFDY	Ps.gene
PRMGFSY	Ps.gene
43 nt.	Ps.gene/Unproductive
28 nt.	Ps.gene/Unproductive
35 nt.	Unproductive

3C 22. Unproductive

443-5542

FIG. 10b

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CDR 2 FRAMEWORK 3 CDR 3

KABAT HUMAN VH1

	STSTAYMELRSLRSED	TAVYYCAR	GGGNDHFDY
HACKFQG	RVTIRRHKSTSTAYMELSSLRSED	TAVYYCAR	GSRYGYDCSGYYL
GYAQKFQG	RVTMTRNTSISTATMELSSLRSED	TAVYYCAR	LAHFSGSPVDWDF

KABAT HUMAN VH2

KKQLQPSLKS	RVTISVDTSKNQFSLKLSVTAAD	TAVYYCAR	GGVVPAAIMDV
KS	RVTISVDTSKNQFSLKLSVTAAD	TAVYYCAR	MARYYDFWSGYSAYDY
SLKS	RLSISQDTSRNQFSLRLSSVTAAD	TAVYYCAR	HRNWGSPVHFDY
	ESTSTAYMELSSLRSED	TAVYYCAR	DSYGQYGGHY

KABAT HUMAN VH3

ISYITSSSSYTNVADSVKG	RFTISRDNKNSLYLQNSLRADDT	TAVYYCAR	DGRFGTYSPSDY
SVKG	RFTISRDDSKSLAYLQVNSLKTED	TAVYYCTR	TIYYDSSGYPYW
YADSVKG	RFTISRDNKNSLFLOHSSLRAEDT	TAVYYCAR	GIALDAFDI
YYADSVRO	RFTISRDNKNSLYLQNSLRADDT	TAVYYCAK	53 NT. UNPROD REARR
DSVKG	RFTISRDNKNSLYLQNSLRDEDT	TAVYYCAR	DHSGTGGGSGSYF
VSAISGSGGSTYYADSVKG	RFTISRDNPKNTLYLQNSLRSED	TAVYYCAR	KDNLWFD
AVISYDGSNKYYADSVKG	RFTISRDNKNTLYLQNSLRADDT	TAVYYCAR	DLGGRGVVVVPAPGGRSIIYYGMDV
GAVISYDGSNKYYADSVKG	RFTISRDNKNTLYLQNSLRADDT	TAVYYCAS	LEGIGTIYYGMDV
	AKNSLYLQNSLRADDT	TAVYYCVR	DDSSSWPKHFQ
QYAAASVKG	RFTISRDDSKNSLYLQNSLNTED	TAVYYCVR	SGVVPYLDY

KNOWN FAMILY

AVYYCAR	DPRIAAAPDYYYYMDV
TAVYYCAR	GAEVVEFTARYYYGLNV

FIG. 11

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FR1	CDR1	FR2
YFTT	SYGSS	WVYSGFWRCLEWNG
GEKPGSSVKV/SCKASGYTFT	DYFMN	WVRQAPGQGLEWMG
QVQLQETIGPRGGEASETTLSTLCVAVSGDSIS	SGNW*1	WVRQPPGKGLEWIG
QVQLQESGPGLVK*SETTLSTCTVSGGSIS	SYVWS	WIRQPPGKGLEWIG
GYTFT	NYCMH	WVRQDHAQGLEWMG
QVQLQESGPGLVKpSETTLSTLCVAVSGDSIS	SGNW*1	WVRQPPGKGLEWIG
GPRLGEASETTLSTCTVSGGSIS	SSSYW	WIRQPPGKGLEWIG
QVQLQESGPGLVKpSETTLSTCTVSGGSIS	SYVWS	WIRQPPGKGLEWIG
LSLCAVSGSSIS	SGNW*1	WVRQPPGKGLEWIG
SETTLSTCAVYGGSF	GYVWS	WIRQPPGKGLEWIG
QVQLVQSGAEVKGPQASVKV/SCKASGYTFT	NYCMH	WVRQVLAQGLEWMG
SETTLSTLCVAVSGDSIS	SGNW*1	WVRQPPGKGLEWIG
SRAQTGEASETTLSTCTVSGGSIS	SSSYW	WIRQPPGKGLEWIG
CPLTCTVSGGSVSSGS	YYWS	WIRQPPGKGLEWIG
GLVKPSETTLSTCTVSGGSIS	SYVWS	WIRQPPGKGLEWIG
SFETTLSTLCVAVSGDSIS	SGNW*1	WVRQPPGKGLEWIG
QVQLVQSGAEVKGPQASVKV/SCKASGYTFT	SYAIS	WVRQAPGQGLEWMG
QVQLQQWAGALLKpSETTLSTCAVYGGSF	GYVWS	WIRQPPGKGLEWIG
QVQLQESGPGLVKpSETTLSTCTVSGGSIS	SSSYW	WIRQPPGKGLEWIG
SPGLVKPSETTLSTCTVSGGSIS	SGGYWS	WIRQPPGKGLEWIG

* indicates stop codon (unsure as sequence remains in frame)
 • sequence terminates due to internal restriction site
 lower case denotes frame shift

FR1	FR2	CDR3
WISAYNGNTNYAQKLGQ	RVTTTDTSTSTAYMELSLRSEDVAVYYCAR	DTVSS
WISAGNGNTKYSQKLGQ	RVTTTDTSTASTAYMQLSSLRSEDVAVYYCAR	DTVSS
EIHHSGSTYYNPSLKS	RVTTTSDTSTKQNFYKLSS•	
RVTTTSGSTNYNPSLKS	RVTTTSDTSTKQNFSLKLSSVTAADTAVYYCAR	DTVSS
LVCPSDGSTSYAQKFGA	RVTTTDTSTSMSTAYMELSSLRSEDVAVYYCAR	DTVSS
EIHHSGSTYYNPSLKS	RVTTTSDTSTKQNFYKLSS•	
EIHHSGSTNYNPSLKS	RVTTTSDTSTKQNFSLKLSS•	
YIYYSGSTNYNPSLKS	RVTTTSDTSTKQNFSLKLSS•	
EIHHSGSTYYNPSLKS	RVTTTSDTSTKQNFYKLSS•	
EIHHSGSTNYNPSLKS	RVTTTSDTSTKQNFSLKLSSVTAADTAVYYCAR	DTVSS
LVCPSDGSTSYAQKFGA	RVTTTDTSTSMSTAYMELSSLRSEDVAVYYCAR	DTVSS
EIHHSGSTYYNPSLKS	RVTTTSDTSTKQNFYKLSS•	
SIYYSGSTYYNPSLKS	RVTTTSDTSTKQNFSLKLSS•	
YIYYSGSTNYNPSLKS	RVTTTSDTSTKQNFSLKLSSVTAADTAVYYCAR	DTVSS
RVTTTSGSTNYNPSLKS	RVTTTSDTSTKQNFSLKLSS•	
EIHHSGSTYYNPSLKS	RVTTTSDTSTKQNFYKLSS•	
RIIPILGIANYAQKFGQ	RVTTTADKSTSTAYMELSSLRSEDVAVYYCAR	DTVS
EIHHSGSTNYNPSLKS	RVTTTSDTSTKQNFSLKLSS•	
EIHHSGSTNYNPSLKS	RVTTTSDTSTKQNFSLKLSS•	
YIYYSGSTYYNPSLKS	RVTTTSDTSTKQNFSLKLSSVTAADTAVYYCAR	DTVSS

FIG. 12

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pSW1

HindIII site AAGCTT

GCATGCAAAATTTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCGTACGGCAGCC
 10 20 30 40 50 60

M K Y L L P T A A
 A G L L L L A A Q P A M A Q V Q L Q E S
 GCTGGATGTTTATTACTGCGTGGCCCAACCGATGGCCCGAGGTGCAGCTGCAGGAGTCA
 70 80 90 100 110 120

G P G L V A P S Q S L S I T C T V S G F
 GGACCTGGCGTGGTGGCGCGCTGCAGAGCGCTGTCCATCACAAGCACCGTCTCAGGGTTC
 130 140 150 160 170 180

S L T G Y G V N W V R Q P P G K G L E W
 TCATTAAAGCGGTTATGGTGTAACTGGTTCGCCAGCGCTCCAGGAAGGGTCTGGAGTGG
 190 200 210 220 230 240

L G M L W G D G H T D Y N S A L K S R L
 CTGGGAATGATTTGGGGTGTATGGAACACAGACTATAATTCAGCTCTCAAATCCAGACTG
 250 260 270 280 290 300

S I S K D N S K S Q V F L K M N S L H T
 AGCATCAGCAAGGACAACTCCAGAGCCCAAGTTTTCTTAAAAATGAACAGTCTGCACACT
 310 320 330 340 350 360

D D T A R Y Y C A R E R D Y R L D Y W G
 GATGACACAGCCAGGTACTACTGTGCCAGAGAGAGAGATTATAGGCTTGACTACTGGGGC
 370 380 390 400 410 420

Q G T T V T V S S SmaI
 CAAGGCACCACGGTCACCGTCTCTCTCATAATAAGAGCTATCGGGGCTAAGCTCGAATTC
 430 440 450 460 470 480

FIG. 13

SUBSTITUTE SHEET

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M K Y L L P T A A

A G L L L A A C P A M A Q V Q L Q E S
 GCTGGATGTTACTCGCTGCCCAACCAGCGATGGCCAGGTGCAGCTGCAGGAGTCA
 70 80 90 100 110 120

S L T G Y G V N W V R C P P G K G L E W
TCA.TTAACCGGGCTATGGTCTAAACTGGTCTGGCCAGCTTCCAGGAAAGGGTCTGGAGTGG
190 220 250 280 310 340

S T S K D N S K S Q V F L K M N S L H T
AGCATTCACGAAGGACAACTCCAGAGCCCAAGTTTCTTTAAATGAACAGTCTGCACACT
310 320 330 340 350 360

Q G T T V T V S S
CAAGGCACACGGTACCGGTCTCTCTATATAGAGCTCGAATTCGCCAAGGTTGCATGC
430 440 450 460 470 480

L L L L A A Q P A M A D I V I T Q S P A
 TTGTTATTACTCGGTGCCCAACCAACGATGGCCGACATCGTCTCTGACTCAGTCTCCAGCC
 550 560 570 580 590 600

H N Y L A W Y Q Q K Q G K S P Q L L V Y
CACAATTATTAGCATGTTATCTCAGAAACAGGGAAAATCTCTCTCAGCTCTCGGTCTA
670 680 690 700 710 720

FIG. 14a

FIG. 14 b

Handwritten: 514 AGCT

Polylinker
TCTAGA GTCGAC CTCGAG
XbaI SalI XhoI

FIG. 15

SUBSTITUTE CHECK

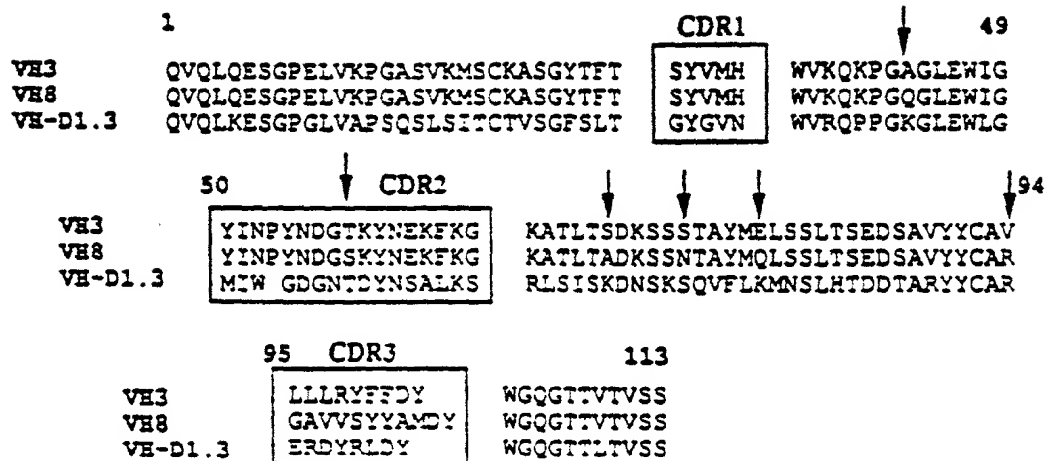


FIG. 16

FR1	QVQLQESGGGLVQPGGSLRLSCAASGFTFS	
	SYAMS	CDR1
FR2	WVRQAPGKGLEWVS	
	AISGSGGSTYYADSVKG	CDR2
FR3	RFTISRDN SKNTLYLQMNSLRAEDTAVYYCAM	
	WRGIATPVSF DLGYFDY	CDR3

FIG. 17

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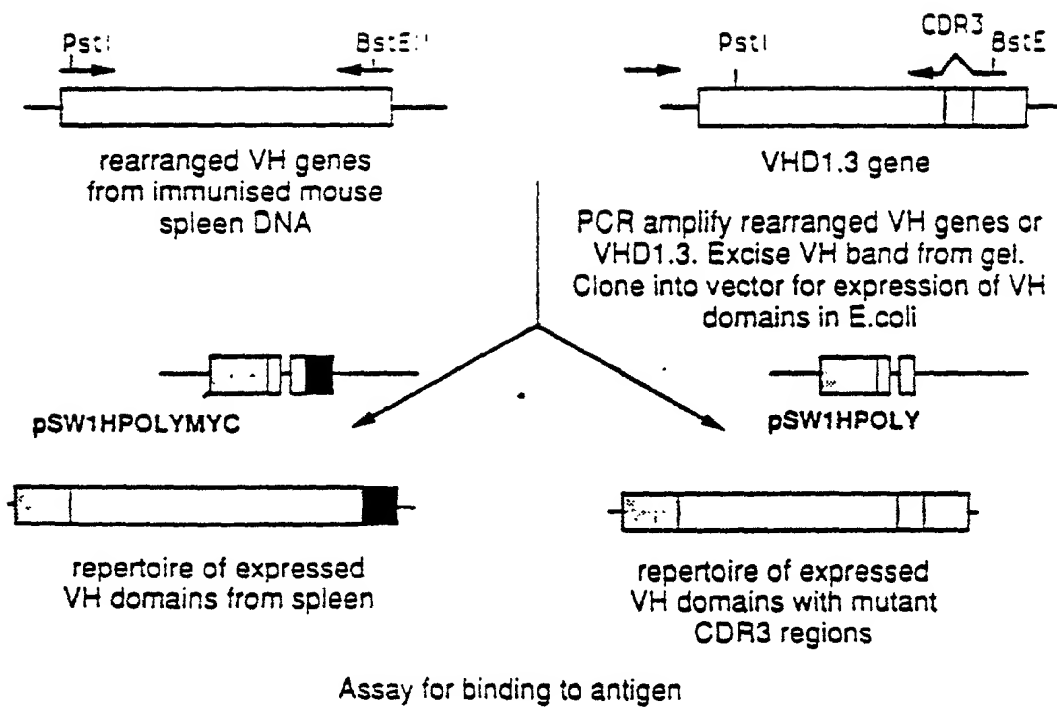


FIG. 18

[illegible]

HLNCHII AAGCTT

A G L L L L A A Q P A M A Q V Q L Q
GUTGGATTGTATTACTGGCTGCCCAACCAGCGATGGCCAGGTGCAGGTGCAG
70 80 90 100 110 PstI

V T V S S
GGTCACGCTCTCTCTCATAAATAAGAGCTGGAAATGGCCAGCTTGCATGC
Bst-III 430 440 450 460 470 480

M K V L L P T A A A G
AAATTCTATTTCAGGAGACAGCTCATATGAATACCTATGGCTACGGCAGCCGCTGGA
490 500 510 520 530 540

T T G T T A T T A C T C G C T G C C C A A C C A G C G A T G G C G A T C G T C C T G A C T C A G T C T C C A G C C
 550 560 570 580 590 600

S L S A S V G E T V T T T C R A S G N I
TGGCTTTGCTGCTGTGTGGAGAAATGTACCATACATGTCGAGCAAGTGGGAATATT
610 620 630 640 650 660

H N Y L A W Y Q Q K Q G K S P Q L L V Y
CAGCATTTATTTAGCATGGTATCAGCAGAAAAGGGGAAATCTCTCTCAGCTCTCTGGTCTAT
670 680 690 700 710 720

Y T T T Z A D G V P S R F S G S G S G T
TATACAAACACCTTAGCAGATGGGTGTGCCATCAAGGTTCACTGGCAGTGGATCAGGAACA
730 740 750 760 770 780

Q Y S L K I N S L Q P E D F G S Y Y C Q
CAATAATCTCTCAAGATCAACAGCCTGCAACCTGAAGATTTGGGAGTTATTACTGTCAA
790 800 810 820 830 840

H F W S T P R T F G G G T K L E I K R
CATTTTGGAGTACTCCTCGGACGTTGGTGGAGGCACCAAGCTGGAAATCAAACGGTAA
850 860 870 880 890 900

TAAGAGCTCGAATTC
91C

FIG. 19

Figure 1 consists of 12 subplots, labeled (a) through (l), each showing a histogram of 1000 simulated values for a specific parameter. The parameters and their corresponding y-axis scales are as follows:

- (a) $\gamma_{\text{transmission}}$: scale 0.0 to 0.2
- (b) $\gamma_{\text{infection}}$: scale 0.0 to 0.2
- (c) γ_{recovery} : scale 0.0 to 0.2
- (d) γ_{death} : scale 0.0 to 0.2
- (e) $\gamma_{\text{quarantine}}$: scale 0.0 to 0.2
- (f) $\gamma_{\text{isolation}}$: scale 0.0 to 0.2
- (g) $\gamma_{\text{hospitalization}}$: scale 0.0 to 0.2
- (h) $\gamma_{\text{ventilator}}$: scale 0.0 to 0.2
- (i) γ_{ICU} : scale 0.0 to 0.2
- (j) γ_{O2} : scale 0.0 to 0.2
- (k) γ_{NIV} : scale 0.0 to 0.2
- (l) γ_{ECMO} : scale 0.0 to 0.2

The x-axis for all plots represents the parameter value, with labels varying by plot to indicate the range of values. The histograms show the frequency of simulated values, with most distributions being roughly bell-shaped and centered around a specific value.

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[illegible]

FIG. 21a

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T G V K T Y N G A L G V D I H E K D H P
ACCGGTGTCAAAACCTATAACGGCGCGCTGGGCGTCGATATTCACGAAAAAGATCACCCA
790 800 810 820 830 840

T I L E M A K A A G L A T G N V S T A E
ACGATTCTGGAAATGGCAAAAGCGCAGGTCTGGCGACCGTAACGTTTCTACCGCAGAG
850 860 870 880 890 900

L Q D A T P A A L V A H V T S R K C Y G
TTGCAGGATGCCACGCCCGCTGGGCTGGGACATGTGACCTCGCGCAAAATGCTACGGT
910 920 930 940 950 960

P S A T S E K C P G N A L E K G G K G S
CCGAGCGCGACCACTGAAAAATGTCCGGTAACTCTGCGAAAAAGCGGAAAAGGATCG
970 980 990 1000 1010 1020

I T E Q L L N A R A D V T L G G G A K T
ATTACCGAACAGCTGCTTAACGCTGCGGACGTTACGCTTGGCGGGCGGCAAAAAC
1030 1040 1050 1060 1070 1080

F A E T A T A G L W Q G K T L R E Q A Q
TTTGCTGAAACGGCAACCGCTGGTGAATGGCAGGGAAAAACGCTGCGTGAACAGGCACAG
1090 1100 1110 1120 1130 1140

A R G Y Q L V S D A A S L N S V T E A N
GGCGCTGGTTATCAGTTGGTGAAGGATGCTGCTCACTGAATTCGGTGACGGAAGCGAAT
1150 1160 1170 1180 1190 1200

Q Q K P L L G L F A D G N M P V R W L G
CAGCAAAAACCCCTGCTTGGCTGTTTGGTGAAGGATGCTGCTCACTGAATTCGGTGACGGAAGCGAAT
1210 1220 1230 1240 1250 1260

P K A T Y H G N I D K P A V T C T P N P
CCGAAAGCAACGTACCATGGCAATATCGATAAGCCCGCAGTCACCTGTACGCCAAATCCG
1270 1280 1290 1300 1310 1320

Q R N D S V P T L A Q M T D K A I E L L
CAACGTAATGACAGTGTACCAACCCCTGGCGCAGATGACCGACAAAGCCATTGAATTGTTG
1330 1340 1350 1360 1370 1380

S K N E K G F F L Q V E G A S I D K Q D
AGTAAAAATGAGAAAGGCTTTTCTGCAAGTTGAAGGTGCGTCAATCGATAAACAGGAT
1390 1400 1410 1420 1430 1440

H A A N P C G Q I G E T V D L D E A V Q
CATGCTGCGAATCCTTGTGGGCAAAATGGCGAGACGGTCGATCTCGATGAAGCCGTACAA
1450 1460 1470 1480 1490 1500

R A L E F A K K E G N T L V I V T A D H
CGGGCGCTGGAATTCGCTAAAAAGGAGGTAACACGCTGGTCATAGTCACCGCTGATCAC
1510 1520 1530 1540 1550 1560

FIG. 21b

000217 19022460

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A H A S Q I V A P D T K A P G L T Q A L
GCCCACGCCAGCCAGATTGTTGCGCCGGATACCAAAGCTCCGGGCCTCAGCCAGGCGCTA
1570 1580 1590 1600 1610 1620

N T K D G A V M V M S Y G N S E E D S Q
AATACCAAAGATGGCGCAGTGATGGTGATGAGTTACGGGAACTCCGAAGAGGATTACAA
1630 1640 1650 1660 1670 1680

E H T G S Q L R I A A Y G P H A A N V V
GAACATACCGGCAGTCAGTTGCGTATTGCGGCGTATGGCCCGCATGCCGCCAATGTTGTT
1690 1700 1710 1720 1730 1740

G L T D Q T D L F Y T M K A A L G L K *
GGACTGACCGACCGAGACCGATCTCTTCTACACCATGAAAGCCGCTCTGGGGCTGAAATAA
1750 1760 1770 1780 1790 1800

AACCGCGCCCGGGAGTGAATTTTCGCTGCCGGGTGGTTTTTTTGCTGTTAGC
1810 1820 1830 1840 1850

FIG. 21c

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GCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCC
 10 20 30 40 50 60

A G L L L L A A Q P A M A Q V Q L Q E S
 GCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCAGGTGCAGCTGCAGGAGTCA
 70 80 90 100 110 120

G P G L V A P S Q S L S I T C T V S G F
 GGACCTGGCCTGGTGGCGCCTCACAGAGCCTGTCCATCACATGCACCGTCTCAGGGTTC
 130 140 150 160 170 180

S L T G Y G V N W V R Q P P G K G L E W
 TCATTAAACCGGCTATGGTGTAAACTGGGTTCCGAGCCTCCAGGAAAGGGTCTGGAGTGG
 190 200 210 220 230 240

L G M I W G D G N T D Y N S A L K S R L
 CTGGGAATGATTTGGGSGTGATGGAAACACAGACTATAATTGAGTCTCAAATCCAGACTG
 250 260 270 280 290 300

S I S K D N S K S Q V F L K M N S L H T
 AGCATCAGCAAGGACAACCTCAAGAGCCAAAGTTTCTTAAAAATGAACAGTCTGCACACT
 310 320 330 340 350 360

D D T A R Y Y C A R E R D Y R L D Y W G
 GATGACACAGCCAGGTACTACTGTGCCAGAGAGAGATTATAGGCTTGACTACTGGGGC
 370 380 390 400 410 420

Q G T T V T V S S * *
 CAAGGCACCCAGGTCACCGTCTCCTCATAATAAGAGCTATCCCGGGAGCTTGCAATGCAAA
 430 440 450 460 470 480

M K Y L L P T A A A G L
 TTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCCGCTGGATTG
 490 500 510 520 530 540

L L L A A Q P A M A D I E L V D L E I K
 TTATTACTCGCTGCCCAACCAGCGATGGCCGACATCGAGCTCGTCCGACCTCGAGATCAAA
 550 560 570 580 590 600

R E Q K L I S E E D L N * *
 CGGGAACAAAACTCATCTCAGAAGAGGATCTGAATTAATAATGATCAAACGGTAATAAG
 610 620 630 640 650 660

GATCCAGCTCGAATTC
 670

FIG. 22

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Q V Q L Q E S G P G L V Q P S Q S L S I
 CAGGTGCAGCTGCAGGAGTCAGGACCTGGCCTAGTGCAGCCCTCACAGAGCCTGTCCATC
 10 20 30 40 50 60

T C T V S G F S L T S Y G V H W V R Q S
 ACCTGCACAGTCTCTGGTTTCTCATTAACTAGCTATGGTGTACACTGGGTTTCGCCAGTCT
 70 80 90 100 110 120

P G K G L E W L G M I W G D G N T D Y N
 CCAGGAAAGGGTCTGGAGTGGCTGGGAATGATTGGGGTGATGGAAACACAGACTATAAT
 130 140 150 160 170 180

S A L K S R L S I S K D N S K S Q V F L
 TCAGCTGTCAAATTCAGACTGAGCATCAGCAAGGACAACCTCCAAGAGCCAAGTTTTCTTA
 190 200 210 220 230 240

K M N S L H T D D T A R Y Y C A R E R D
 AAAATGAACAGTCTTGCACACTGATGACACAGCCAGGTACTACTGTGCCAGAGAGAGAGAT
 250 260 270 280 290 300

Y R L D Y W G Q G T T V T V S S
 TATAGGCTTGACTACTGGGGCCAGGGACCCAGGTCAACCGTCTCTCTCA
 310 320 330 340

FIG. 23

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

I, the below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled Single domain

ligands, receptors comprising said ligands methods for their production and use of said
the specification of which (check applicable box(es)): ligands and receptors

☐ is attached hereto.

☐ was filed on _____ as U.S. Application Serial No. _____

☒ was filed as PCT international application No. PCT/GB89 / 01344 on 13th November 1989 Designating U.S.A.

and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 C.F.R. 1.56(a). I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Prior Foreign Application(s):

Application Number	Application Number	Country	Day/Month/Year Filed	Day/Month/Year Filed
1) 8826444.5	6) 8913900.0	U.K.	1) 11 11 1988	6) 16 06 1989
2) 8906034.7	7) 8918543.3	U.K.	2) 16 03 1989	7) 15 08 1989
3) 8909217.5		U.K.	3) 22 04 1989	
4) 8911047.2		U.K.	4) 15 05 1989	
5) 8912652.8		U.K.	5) 02 06 1989	

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56(a) which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s):

Application Serial No.

Day/Month/Year Filed

Status: patented,
pending, abandoned

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And I hereby appoint Nixon & Vanderhye P.C., 2200 Clarendon Boulevard, 14th Floor, Arlington, Virginia 22201, telephone number (703) 875-0400 (to whom all communications are to be directed), and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009.

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FOR ADDITIONAL INVENTORS, check box ☐ and attach sheet with same information and signature and date for each.